PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
M. Bristow *et al.*

Serial No.: 09/558,472

Filed: April 25, 2000

For: DIAGNOSIS AND TREATMENT OF MYOCARDIAL FAILURE

Group Art Unit: 1632

Examiner: Ram R. Shukla

Atty. Dkt. No.: MYOG:004USD1/SLH

Confirmation No.: 8819

CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

April 20, 2007
Date

Steven L. Highlander

SUBSTITUTE APPEAL BRIEF

TABLE OF CONTENTS

Page

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: M. Bristow *et al.*	Group Art Unit: 1632
Serial No.: 09/558,472	Examiner: Shukla, Ram R.
Filed: April 25, 2000	Atty. Dkt. No.: MYOG:004USD1/SLH
For: DIAGNOSIS AND TREATMENT OF MYOCARDIAL FAILURE	

APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This Substituted Appeal Brief is filed in response to the Notice of Non-Compliant Appeal Brief (37 CFR 41.37) mailed on March 28, 2007, to which a response is due on April 28, 2007. No fees are believed due in connection with this filing. However, should any fees be due, applicants authorize the Commissioner to debit Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/MYOG:004USD1.

I. Real Party In Interest

The real parties in interest are the assignee, University Technology Corporation, University of Colorado and the licensee, Myogen, Inc.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

Claims 1-26 were filed with the application. Claims 1-22, and 24-26 have been canceled. Thus, claim 23 is pending, stands rejected, and is appealed. A copy of the pending claim is attached.

IV. Status of the Amendments

All amendments have been registered and examined.

V. Summary of the Claimed Subject Matter

The present invention is drawn to a method of treating myocardial failure in a human comprising administering an effective amount of a transgene encoding for α-MHC, wherein expression of α -MHC provides improvement in left ventricular ejection fraction. Specification at page 4, lines 23-27, and pages 10-13.

VI. Grounds of Rejection to be Reviewed on Appeal

Is claim 23 enabled under 35 U.S.C. §112, first paragraph?

VII. Argument

A. Summary

The rejection under 35 U.S.C. §112, fist paragraph, is based on the premise that the specification is defective in providing an adequate basis for teaching or providing guidance with respect to how to deliver α-MHC via a transgene, that the specification exemplifies a transgenic animal that fails to extrapolate to treating a human with a transgene, and that the specification fails to provide guidance for what specific levels of α-MHC would be therapeutic.

However, the rejection is not founded upon a proper understanding of the science, nor does the examiner appear to understand the relevance of the last reference supplied by appellants (abstract from the lab of Jeffrey Robbins). Transgenic models, especially the rabbit model used by Robbins, are not only instructive, but they can be fashioned to mimic the human state, and thus are highly informative and give more than adequate guidance for treating the human disease. The Robbins reference, coupled with what is now known about α-MHC, shows very specifically what levels of α-MHC would be cardioprotective.

Finally, the references supplied by appellants clearly show that at the time of filing delivery of genes directly to the heart, a method disclosed in the specification, was not only possible, it was considered practicable. The enablement rejection is therefore improper because the specification, coupled with references filed both before and after filing, is sufficient to supply "suitable proofs" as required by law that the invention is indeed enabled.

B. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994.

Dickinson v. Zurko, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

C. Rejection Under 35 U.S.C. §112, First Paragraph

Claim 23 stands rejected as lacking an enabling disclosure in the specification. According to the examiner, the specification is defective in (a) failing to provide an adequate basis for predicting that an increase in α-MHC transcripts would benefit subjects having myocardial failure, (b) failing to provide correlation of α-MHC transgene expression *in vivo* with therapeutic benefit, (c) failing to teach or provide guidance with respect to specific levels of α-MHC that would be therapeutic, and (d) failing to teach or provide guidance for delivery of the α-MHC transgene to the heart. Appellants have provided extensive responses to the examiner's concerns, and for the most part, the examiner has simply "reiterated" the PTO's previous positions. Therefore, appellants appeal the examiner's final position.

In their last response, appellants directed the examiner to the enclosed poster abstract from the lab of Jeffrey Robbins, Ph.D., presented at the Keystone Meeting on "Biology of Cardiac Disease" held on March 7, 2004. This data evolved from Robbins' findings on the rabbit heart model presented in James *et al.*, *JMCC* 34, 873-882 (2002). As shown in this

abstract, directly supporting the inventor's paradigm and abrogating the examiner's concerns regarding this invention, addition of α-MHC transcripts via a transgenic approach strengthens the heart and renders it resistant to tachycardia-induced cardiomyopathy. Robbins' study is highly applicable to the human setting because Robbins took advantage of the fact that the rabbit heart is constituted much like the human heart, expressing low levels of α-MHC when healthy, and then expressing virtually no α-MHC when the heart is diseased. In this model, if an increase in α-MHC could be shown to be protective, it would be highly predictive for what would be seen in the human heart. His abstract provides proof of concept for the rejected claims, showing that adding even as little as 10-15% more α-MHC than baseline is protective to the heart. In other words, and exactly as claimed by the current inventors, adding an α-MHC transgene causes an increase in LVEF directly related to the additional α-MHC expressed in heart tissue.

The examiner found this data to be inconclusive, focusing on the fact that the animal used was a transgenic animal. The examiner's concerns exhibit a failure to understand the technology at hand. The α-MHC promoter is ***only*** expressed in the heart. Therefore, whether or not the gene is present in every cell in the body is completely irrelevant. This particular model is a perfect representative model for the human disease because the gene is expressed where an exogenous transgene would be expressed, it is expressed in a measurable amount which provides guidance on a therapeutic level to clinicians, and it is the exact same gene that would be used in the claimed method. Thus, the examiner's concerns about the use of a transgene are clearly out of touch with the actual science being used and cannot stand up in the face of a true understanding of the relevance of the Robbins data.

While the Robbins data alone should be sufficient to overcome the examiner's concerns and enable this claim, appellants will address the other issues raised by the examiner's earlier

rejections. Appellants have previously supplied the examiner with a variety of references regarding gene therapy (see response mailed May 19, 2004), but the examiner has focused almost exclusively on the Jones reference, arguing it was not dispositive. The final rejection ignored the numerous other references supplied (and again referenced below) by appellants regarding the enablement of cardiac gene therapy, and in the examiner's most recent communication (dated July 9, 2004) these references have been found to be ineffectual in supporting the method as claimed. In a previous response (dated Sept. 11, 2001), appellants provided a number of publications, far more relevant than those cited by the examiner in his responses, reporting on the successful transfer of genes into cardiac tissue. Alexander *et al.*, *Clin. Exp. Pharmacol. Physiol.*, 26:661-668 (1999) reported gene transfer into myocardium through direct injection of plasmid DNA and viral transfer. Chien *et al.*, WO/2000/15821 describe the use of recombinant adenovirus-mediated expression of transgenes in both neonatal and mature cardiac tissues. Other papers reporting cardiac transgene expression included Davidson *et al.*, *Circulation* 104:131 (2001), Pachucki *et al.*, *Endocrinology* 142:13 (2001), Shinmura *et al.*, *Japan Heart J.* 41:633 (2000), Silva *et al.*, *FASEB* 14:1858 (2000), Lenhart *et al.*, *Am. J. Physiol. Heart Circ. Physiol.* 279:H986 (2000), Lazarous *et al.*, *Cardiovasc. Res.* 44:294 (1999), and Wickenden *et al.*, *Circ. Res.* 85:1067 (1999).

Additionally, the examiner has repeatedly failed to address other references provided by appellants that further undercut the examiner's position on enablement. In particular, the examiner has failed to convincingly rebut the claims made by Yue, Schroeder, O'Donnell, del Monte, and Fromes. Fromes *et al*, *Gene* Therapy, 12:683-688 (1999) described the successful delivery of a gene to the myocardium by intraperitoneal injection. The Fromes paper states that "gene therapy is a potential new strategy for cardiovascular diseases" and adds that "several

studies have demonstrated the feasibility of gene transfer into the heart muscle." In addition to Fromes, appellants referenced a number of previous articles that demonstrated the potential of direct injection of genes into the myocardium (*see* Lin *et al*, *Circulation*, 82:2217-2221 (1990); Stratford-Perricaudet *et al*, *J. Clin. Invest.*, 90:626-630 (1992); Von Harsdorf *et al.*, *Circ. Res.*, 72:688-695 (1993); French, Circulation, 90(5) 2414-2424, (1994); Lee *et al.*, *J. Thorac. Cardiovasc. Surg.*, 111:246-252 (1996); Coffin *et al.*, *Gene Therapy,* 3:560-566 (1996); and Kypson *et al.*, *J. Thorac. Surg.*, 115:623-630 (1998)). Fromes, however, constituted an advance over those reports in developing a technique for "local delivery of the therapeutic gene into the pericardium," demonstrating the successful delivery of a gene to the heart. Fromes' results proved that "intra-pericardial injection ... leads to an efficient and safe strategy to deliver a transgene to the heart." The successful approach taken by Fromes came on the heels of another successful application of cardiovascular gene therapy by Hajjar *et al*, *Proc. Natl. Acad. Sci. USA*, 95:5251-5256 (1998). Hajjar used a catheter-based technique to successfully alter cardiac function in rat hearts. This study was seen as opening the prospect "of using somatic gene transfer to modulate overall cardiac function *in vivo*."

Appellants have also discussed how later researchers built on the success seen by both Fromes and Hajjar, generating additional data that validated the feasibility and effectiveness of cardiovascular gene therapy. Schroeder *et al.*, *Transplantation*, 70:191-198 (2000) showed that addition of anti-CD4 monoclonal antibodies improved gene transfer into rat cardiac grafts. O'Donnell *et al.*, *Circ. Res.*, 88:415-421 (2001) showed that sarcoplasmic reticulum (SR) ATPase (SERCA), could be expressed in cardiac myocytes. del Monte *et al.*, *Circulation*, 104:1424-1429 (2001) also showed effective transfer of and expression of SERCA2a into a rat heart through adenoviral gene transfer. Li *et al.*, *Gene. Ther.*, 21:1807-1813 (2003) showed that

an adenoviral associated vector (AAV) could be successfully used to transfer a reporter gene and a therapeutic gene into the heart of a hamster. Appellants have pointed to Yue *et al.*, *Circulation* (2003), who went yet a step further and actually treated a cardiovascular disease using an AAV vector to deliver a therapeutic gene to the heart of a diseased mouse. Yue not only successfully delivered the gene to the heart, but was able to see improvement of cardiovascular function and further saw improvement in disease state. Finally, appellants directed the examiner to the statements of Poller *et al.*, Z Kardiol., 93(3):171-93 (2004), a recent publication exploring cardiac gene therapy approaches. Pollard conclusively states "the important goal of cardiac long-term stability of therapeutic vectors has recently been achieved in animal models using vectors derived from adeno-associated viruses (AAVs)."

In his last communication, the examiner argued that the methods disclosed by all these papers (an argument made without any direct refutation of the specific references or the conclusions made by appellants) could not be used to enable the invention, which is contrary to the law and appears to now elevate the rejection to the level of requiring a working model. According to MPEP §2164.02 "an applicant need not have actually reduced the invention to practice prior to filing." It is important to remember that "because only an enabling disclosure is required, appellant need not describe all actual embodiments. The absence of working examples will not by itself render the invention non-enabled." MPEP §2164.02. Furthermore, nowhere in patent law is it stated that the use of post-filing references is prohibited from enabling a claimed method. In fact, such practice is commonplace and accepted, as the "court has approved use of later publications as evidence of the state of art existing on the filing date of an application" (*In re Hogan*, 559 F.2d 595 (CCPA, 1977), recently upheld in *Plant Genetic Sys. v. Dekalb Genetics Corp.*, 315 F.3d 1335 (Fed. Cir., 2003)). As such, the examiner's assertion that "none of the post

filing arts followed the method taught by the specification," is not relevant because the claimed method is generic to treating the disease and the methods disclosed by the cited references all fall within the body of art claimed by the current invention. Thus, they should satisfy as examples to enable the claimed invention.

Appellants therefore submit that the overwhelming body of evidence in the literature supports gene therapy in the heart as an enabled technology and the present record provides adequate evidence for the value of α-MHC therapy. In further response to the examiner's statement that many of the above-cited references are after the date of filing, appellants cite to *In re Marzocchi*, 169 USPQ 370 (CCPA 1971), stating that an enablement rejection "can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling." The cited references, coupled with the new abstract by Robbins, are indeed the "suitable proofs" required by the law, and nowhere in the law is it required that these proofs be in the evidentiary record prior to the date of filing.

In sum, appellants submit that the present record provides adequate evidence of the value of α-MHC therapy. Therefore, reversal of the rejection is respectfully requested.

D. Conclusion

In light of the foregoing, appellants respectfully submit that all pending claims are definite and supported by the application as filed. Therefore, it is respectfully requested that the Board reverse each of the pending rejections.

Respectfully submitted,

Steven L. Highlander
Reg. No. 37,642

Fulbright & Jaworski L.L.P.
600 Congress Ave., Suite 2400
Austin TX 78701
512-474-5201

Date: April 20, 2007

VIII. APPENDIX A – APPEALED CLAIMS

1-22. (Canceled)

23. (Previously presented) A method of treating myocardial failure in a human comprising administering an effective amount of a transgene encoding for α-MHC, wherein expression of α-MHC provides improvement in left ventricular ejection fraction.

24-26. (Canceled)

IX. APPENDIX B – EVIDENCE CITED

Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994
Dickinson v. Zurko, 527 U.S. 150, 158 (1999)
In re Gartside, 203 F.3d 1305, 1315 (Fed. Cir. 2000)
In re Hogan, 559 F.2d 595 (CCPA, 1977)
Manual of Patent Examining Procedure §2164.02
In re Marzocchi, 169 USPQ 370 (CCPA 1971)
Plant Genetic Sys. v. Dekalb Genetics Corp., 315 F.3d 1335 (Fed. Cir., 2003)
Exhibit A – James – submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit B – Alexander - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit C – Chien - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit D – Davidson - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit E – Pachucki - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit F – Shinmura - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit G – Silva - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit H – Lehnart - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit I – Lazarous - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit J – Wickenden - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit K – Fromes - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit L – Lin - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit M – Stratford-Perricaudet - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit N – Von Harsdorf - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit O – French - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit P – Lee - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit Q – Coffin - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit R – Kypson - submitted with Information Disclosure Statement Dated October 2, 2003.

Exhibit S – Hajjar - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit T – Schroder - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit U – O'Donnell - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit V – del Monte - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit W – Wang - submitted with Information Disclosure Statement Dated October 2, 2003.
Exhibit X – Poller - submitted with Information Disclosure Statement Dated October 2, 2003.

X. APPENDIX C – RELATED PROCEEDINGS

None

EXHIBIT A

J Mol Cell Cardiol 34, 873–882 (2002)
doi:10.1006/jmcc.2002.2025, available online at http://www.idealibrary.com on IDEAL®

Transgenic Rabbits Expressing Mutant Essential Light Chain do not Develop Hypertrophic Cardiomyopathy

Jeanne James, Yan Zhang, Kathy Wright, Sandra Witt, Elizabeth Glascock, Hanna Osinska, Raisa Klevitsky, Lisa Martin, Karen Yager, Atsushi Sanbe and Jeffrey Robbins

Children's Hospital Research Foundation, Cincinnati, Ohio 45229-3039, USA

(Received 7 February 2002; accepted in revised form 11 April 2002)

J. James, Y. Zhang, K. Wright, S. Witt, E. Glascock, H. Osinska, R. Klevitsky, L. Martin, K. Yager, A. Sanbe and J. Robbins. Transgenic Rabbits Expressing Mutant Essential Light Chain do not Develop Hypertrophic Cardiomyopathy. *Journal of Molecular and Cellular Cardiology* (2002) 34, 873–882. Mutations in multiple sarcomeric proteins can cause familial hypertrophic cardiomyopathy. Although a M149V mutation in the myosin light chain is associated with the human disease, the data from transgenic (TG) mouse models are conflicting. When a human genomic fragment containing the M149V essential myosin light chain was used to generate TG mice, the phenotype was recapitulated. However, when the mouse cDNA containing the mutation was used to generate TG animals, no phenotype could be discerned. TG rabbits can be a valuable complement and extension to mouse-based TG models and we wished to determine whether expression of this mutation in the rabbit heart would result in the disease. The rabbit essential light chain cDNA was isolated, sequenced, the M149V mutation made and the cDNA placed into the β-myosin heavy chain promoter, which efficiently drives cardiac expression in the rabbit ventricles. Multiple TG rabbit lines showing different levels of protein replacement were obtained. No discernible pattern of disease was apparent at the structural or functional levels at either the neonatal, juvenile or adult stages. We conclude that the M149V mutation is not causative for FHC when expressed in the rabbit within the context of the endogenous protein.

Key Words: Transgenesis; Myosin; Gene; Muscle; Rabbit.

Introduction

Manipulating the mouse genome via gene targeting and transgenesis allows one to directly test structure–function and cause-and-effect relationships in cardiovascular function. Although successful TG experiments have been performed in a number of small and large animal species, practically, the mouse has been the animal of choice for laboratory-based cardiovascular studies. There are now literally hundreds of experiments in which cardiac-specific transgenesis has been used to establish structure–function relationships between the presence/absence of a particular protein (or its mutated form) and normal or abnormal function at the molecular, cellular and physiological levels.[1]

However, the mouse heart is fundamentally different from the human heart in terms of its basic motor proteins. While the adult mouse ventricle expresses the α-myosin heavy chain (MHC) isoform, the human ventricle expresses β-MHC,[2] and the mouse heart beats ten times as quickly. The differences in the molecular motor underscore the basic differences between mouse and human, and the cardiovascular consequences of directed mutations that are placed into the mouse either by

Please address all correspondence to: Jeffrey Robbins, Division of Molecular Cardiovascular Biology, MLC 7020, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA. Tel: 513-636-8098; Fax: 513-636-5958; E-mail: jeff.robbins@chmcc.org

0022–2828/02/070873+10 $35.00/0

transgenesis or gene targeting must be applied with caution to large animals or humans. Indeed, recent studies with mouse models of familial hypertrophic cardiomyopathy (FHC) illustrate both the strengths and weaknesses of the mouse models.[3–5]

Mutations in sarcomeric proteins are responsible for FHC, with mutations identified in many components of the cardiac sarcomere.[6] To date, three mutations have been identified in the ventricular isoform of the essential light chain (ELC1v),[7,8] with an unusual phenotype of mid-cavitary obstruction and papillary muscle hypertrophy described in association with the M149V ELC1v mutation.[8] Since ELC1v binds to the neck region of myosin heavy chain (MHC), changes in ELC1v structure presumably alter myosin function, and thus sarcomeric function.

Two mouse models of the M149V mutation, using different TG approaches and possessing divergent phenotypes, have been described.[9,10] In the experiments of Vemuri *et al.*, the entire human mutant genomic DNA locus was expressed in TG mice and produced a mouse cardiomyopathy quite similar to the human ELC1v FHC phenotype. The authors postulated that the ELC1v mutation resulted in an altered stretch-activation response with the end effect of reduced oscillatory power leading to hypertrophy.[10] In contrast, the experiments of Sanbe *et al.* employed a mutated mouse cDNA expressed in TG mice. Despite nearly complete replacement of endogenous mouse ELC1v with the corresponding mutant protein, the mice did not develop mid-cavitary obstruction or hypertrophy.[9] One possible explanation for the different experimental results is that the Vemuri phenotype results in part from a species "mismatch" of human protein in a mouse heart: the ELC1v protein sequences differ between the two species and the predominant MHC isoform is likewise distinct as noted above.

When compared to the mouse, the rabbit heart is more closely related to the human heart at the molecular, biochemical and physiological levels and can be a useful complement to the mouse TG studies. To resolve the mouse data and to test the ability of the M149V mutation to cause disease in a heart with a motor protein complement more similar to the human ventricle, we created multiple lines of TG rabbits in which varying proportions of the normal ELC1v protein were replaced with the ELC1v M149V mutation (M154V in the rabbit sequence). Rabbits from three independent lines were analyzed at the molecular, anatomical and functional levels to determine the cardiac consequences of mutated ELC1v expression. Similar to the results of Sanbe *et al.*, no significant hypertrophic or functional changes resulted from expression of the M154V mutation.

Materials and Methods

ELC1v M154V TG rabbits

Rabbits were housed in an AAALAC approved facility. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. Rabbit ELC1v cDNA was cloned using RT-PCR technology. The product was completely sequenced and the M154V mutation introduced with PCR mutagenesis. A vesicular stomatitis virus (VSV) epitope tag (YTDIEMNRLGK) was placed at the carboxy terminus proximal to the stop codon and the cDNA subcloned into the mouse β-MHC promoter [Fig. 1(a)]. The VSV epitope tag has been used at the carboxy terminus without any detectable effects in previous studies.[9,11] Potential founders were screened by genomic Southern analyses and three founders that transmitted the transgene to their offspring were identified.

Physiologic analyses

The F1 generation was aged 2–12 months before physiologic analysis by echocardiography and/or 5–15 months for cardiac catheterization. After shaving the chest, echocardiography was performed under ketamine sedation using a Hewlett-Packard 5500 Ultrasound System and a 7.5 MHz transducer. Contrast echocardiography was performed using intravenous Optison (Mallinckrodt). Both two-dimensional and M-mode images were recorded on videotape and analyzed off line as described.[12]

Cardiac catheterization was performed after initial sedation with intramuscular ketamine followed by isoflurane anesthesia. Femoral access was obtained via cutdown and a 4 Fr sheath (Cook) placed in the artery. A 4 Fr pigtail (Cook) was advanced into the sheath and positioned in the left ventricle. Pressure measurements and the electrocardiogram were recorded with a Prucka Cardio Lab 4.11 physiologic monitoring system (GE-Marquette). Contrast angiography was performed with Optiray 350 (Mallinckrodt) contrast diluted 1:1 with normal saline using a Liebel-Flarsheim Angiomat Illumena Digital Injection System (Mallinckrodt). Cineagiography was recorded with an OEC Series 9800 Digital Cardiac Imaging System (General Electric) in the left axial oblique and

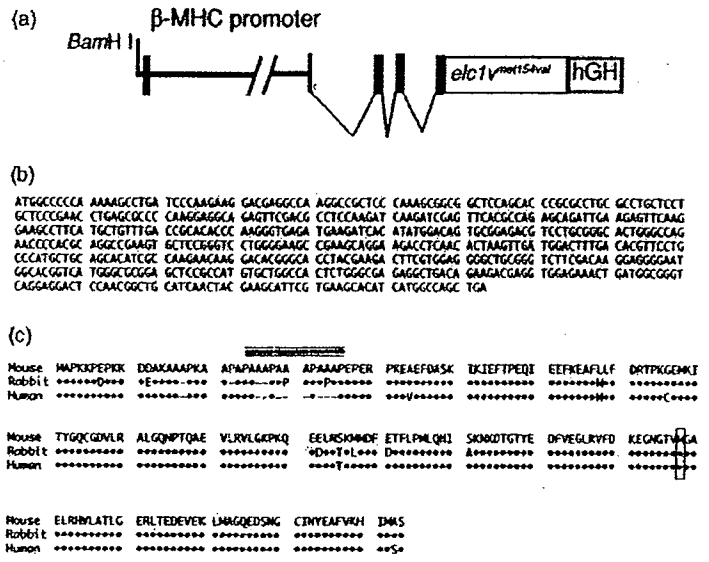


Figure 1 Comparison of the mouse, rabbit and human ELC1v cDNAs. (a) A schematic diagram of the mutant transgenic construct. Solid rectangles indicate the 4 exons of the 5′ untranslated sequence. (b) Coding sequence of rabbit ELC1v. (c) Comparisons of the ELC1v amino acid sequences of mouse, rabbit and human. Dashes were inserted in rabbit and human sequences to maximize alignment. The triple line indicates the alanine-proline rich region and the mutated methionine is boxed.

right axial oblique projections. Following the procedure, the catheters were withdrawn, the femoral artery ligated and the incision closed. The rabbits recovered from anesthesia before being returned to their cages.

Protein and RNA analyses

The rabbits were killed with ketamine and xylazine. The hearts were quickly removed and dissected. Left and right atria (LA and RA), left and right ventricular free wall (LVFW and RVFW), interventricular septum (IVS) and anterior and posterior papillary muscle (APM and PPM) were weighed. Tissue samples were either frozen in liquid nitrogen for molecular analyses or processed for light and electron microscopy as described.[13] RNA expression was determined by Northern and RNA blotting using RNA isolated from LA, RA, LVFW, RVFW, IVS, APM and PPM.[14] Transcript expression was normalized to GAPDH expression with the RA and LA values averaged for atrial expression and the LVFW, RVFW, IVS, APM and PPM values averaged for ventricular expression.

The mutant TG protein was analyzed by subjecting isolated myofibrils (from LA, LVFW, APM and PPM) to SDS-PAGE. Protein was quantified by Western blotting with an anti-ELC1v polyclonal primary antibody made against an 18-amino acid epitope (ERPKEAEFDASKIKIEFT). Primary antibody was used at a dilution of 1 : 50 000 with a horseradish peroxidase conjugated secondary antibody. Quantitation was carried out via by chemiluminescence (Amersham) detection using a STORM 820 (Molecular Dynamics). TG protein replacement was calculated as VSV-tagged ELC1v/(VSV-tagged ELC1v + endogenous ELC1v) and the total level of LV replacement determined by averaging the values for the LVFW, APM, and PPM. Statistical significance was determined by unpaired Student's *t*-test.

Results

The rabbit ELC1v sequence and transgenic replacement

A prerequisite for these studies was to obtain the rabbit cDNA, as we are reluctant to use cross-species contractile proteins in replacement strategy-based experiments. Multiple cDNA clones were isolated and sequenced [Fig. 1(b)]. ELC1v is highly conserved between the mouse, rat, rabbit and human. The most significant difference lies in the amino-terminus where an alanine/proline (A/P)-rich extension in smaller mammals becomes progressively shorter as species size increases [Fig. 1(c)]. Although the exact function of the A/P-rich region is not known, it appears likely that ELC1v modulates cross-bridge kinetics via this region.[15,16] A VSV epitope tag was

added proximal to the stop codon so that TG expression could be easily assayed. The DNA was then placed into the β-MHC promoter construct [Fig. 1(a)] and used to generate TG rabbits.[17,18] Three founders were obtained and used to establish lines 4, 11 and 66. At maturity, lines 4 and 11 demonstrated approximately 1.2-fold overexpression of ELC1v and line 66 1.8-fold overexpression as compared to nontransgenic (NTG) rabbits. Transgene copy number was 2, 7 and 14 diploid copies for line 4, 11 and 66, respectively (data not shown).

Transgenic protein expression was determined by Western blotting. At 6–8 weeks post-birth, line 4 had 29% replacement of endogenous ELC1v with ELC1v M154V and line 11 had 35% replacement. Line 66, the highest expressing line, showed 41% replacement (Fig. 2). Ventricular protein levels in the juvenile and adult (8–12 month) animals were assessed [Fig. 2(b)]. The average level of LV replacement was $24 \pm 5\%$ in line 4, $30 \pm 2\%$ in line 11 and 33 ± 5 in line 66 (mean $\pm$ SD, $P < 0.05$ line 11 *vs* line 4, $P < 0.01$ line 66 *vs* line 4; $P =$ NS for line 11 *vs* line 66). The changes in transgene expression level observed over time as the juveniles mature is a phenomenon we have observed when using the mouse MHC promoters in rabbit and may be caused by position dependent effects on mouse promoter activity as well as subtle developmental modulation of the promoter's transcriptional activation.[17] Clearly though, over a long period of time a significant portion of the ELC1v consists of the mutant TG species.

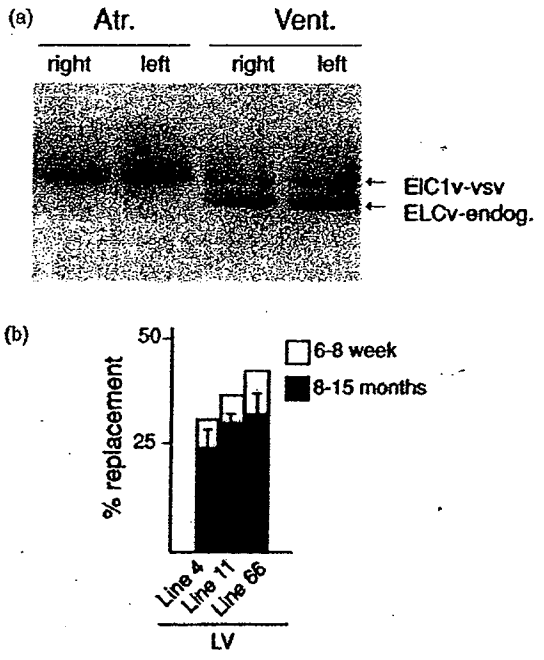


Figure 2 TG protein production. (a) Typical Western blot performed with a polyclonal primary antibody recognizing ELC1v in myofibril extracts from the right atrium (RA) left atrium (LA), right ventricle (RV) and left ventricle (LV) of a 5 week old line 66 rabbit. (b) Western blots as in (a) were performed on myofibril extracts from all three experimental lines and the histograms shown for LV samples at 6–8 weeks (total column height) and at 8–15 months (shaded portion).

Morphometric analysis

To determine if any hypertrophy was taking place, the hearts were subjected to morphometric analysis after the physiological experiments were completed (9–15 months). After dissection, the mass of the RVFW, LV (combined LVFW + IVS), APM and PPM were determined. No changes in body mass or chamber weights could be detected [Figs 3(a), (b)].

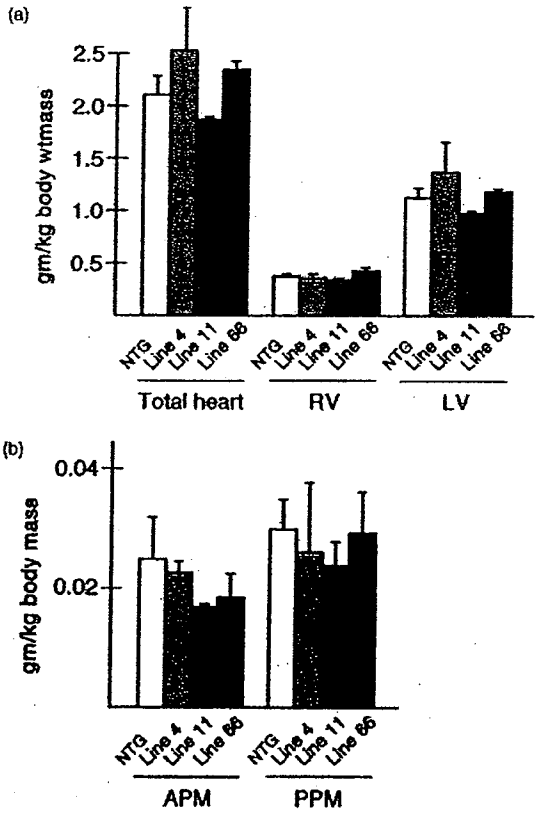


Figure 3 TG hearts do not hypertrophy. (a) Comparison of whole heart, RV and LV mass to body mass in the adult, 1 year old animals. (b) Comparison of anterior and posterior papillary muscle (APM and PPM, respectively) mass to body mass. Data are shown as mean $\pm$ SD. There were no significant differences between the NTG and TG animals in any of the lines.

Molecular markers of hypertrophy

A panel of transcript specific oligonucleotides complementary to the rabbit isoforms of α-MHC (5′-CAGGCACTCGTGTTTATTGCGGGTTAACAAG-AGCGGGGTTC), β-MHC (5′-GCGGA TCAACGC-GTCACCAGGCTATTCCTCATTCAAGCT), ELC1v (5′-CTCTGGCGTGAACTC GATCTTGATCTTGGAG-GCGTCGAACTCTGCC), atrial natriuretic factor (ANF) (5′-CTCA GATAAGGTGCAGTAACGACTCG-ATGCAATGAGATGAGACAAAGTGAC), SERCA2A (5′-AGGTGTGTTGCTAAAAACGCACATGCACGCA-ACGGAACACCCTTACACTTCTGCAAAATG), phos-pholamban (5′-TGACGTGCTTGTTGAGGCATTTC-AATGGTTGAGGCCCTTCTTATAGCAGAGCGAGT-GAGG), cardiac actin (5′-GGTGGCTCGGAAGACTC-CAAGA AGCATAATACTGTCATCCTGAACAC), skeletal actin (5′-GCGAGCAGTGCGTGGGTGTG CGGTGGCAGCAGTGGAAGTTGT) and glyceralde-hyde phosphate dehydrogenase (GAPDH) (5′-CTG-AGGGCCTCTCGTCCTCCTCTGGTGCTCTCGCTG) were designed based on available GenBank se-quences or on sequence obtained in our laboratory. After confirming the specificity for detecting their cognate sequences by genomic Southern analyses, the oligonucleotides were used to determine whether molecular markers of hypertrophy had been activated in the TG hearts. We found no signi-ficant activation of hypertrophy markers in either atria or ventricle of any of the lines in the adult 9–15 month animals. Specifically, β-MHC, ANF and skeletal actin were not up-regulated nor were SERCA2A and phospholamban down-regulated in the TG rabbits compared to NTG controls (data not shown).

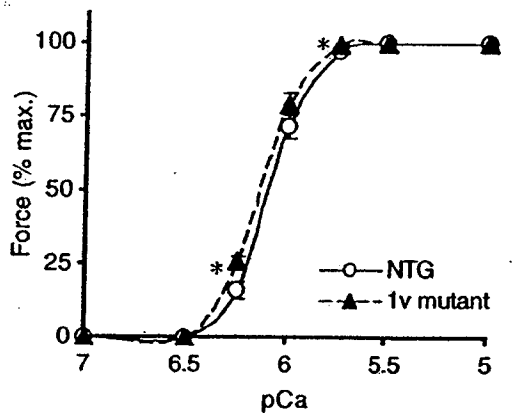


Figure 4 Myofibril function. pCa-force relationship of skinned ventricular fibers from Line 66 rabbits and NTG littermate controls at 6 weeks of age ($n = 3$ for each group). There was a subtle increase in calcium sensitivity in TG fibers. $^*P < 0.05$.

Skinned fiber analysis

To identify any change in myofibril function caused by the M154V mutation, skinned fiber analysis was performed on samples isolated from right ventri-cular papillary muscles of 6 week old Line 66 TG and NTG littermates. Percent replacement of endo-genous ELC1v with the mutant species was con-firmed by Western blotting. In TG animals, we found a very subtle difference calcium sensitivity as evidenced by the pCa-force relationship (Fig. 4). There were no significant differences in unloaded shortening velocity (the "slack test"), maximum shortening velocity or maximum relative power (data not shown).

Structural analyses

Cardiac tissue was examined at both the light (hematoxylin and eosin and trichrome stains) and ultrastructural levels in an attempt to detect any morphological changes due to expression of the mutated ELC1v (Fig. 5). Hearts from adult control and line 66 rabbits were assessed in blinded studies. Some cellular degeneration was evident in both cohorts, which was attributed to the necessary delay in fixation secondary to assessment of cardiac chamber mass. These changes were most pro-nounced at the ultrastructural level, but there were no detectable differences between the control and line 66 rabbits under any staining conditions.

Functional analyses

In order to determine whether any functional changes had occurred, the animals were sedated with ketamine and examined by transthoracic echocardiography. Sixteen rabbits were imaged: seven NTG (mean age 3–13 months), four animals from line 4 (13 months), two animals from line 11 (9 months) and three animals from line 66 (4.5 months) (Table 1). There were no significant differences in interventricular septal thickness, pos-terior wall thickness, left ventricular end-systolic or end-diastolic dimension, shortening fraction, heart rate or papillary muscle dimension. None of the TG rabbits exhibited LV outflow tract obstruction (data not shown). Since FHC is a disease that manifests itself with age, 6 rabbits (3 TG and 3 NTG) from line 11 and 2 rabbits (1 TG and 1 NTG) line 66 were studied at 1 year. No differences were detected.

Cardiac catheterization was performed in rabbits older than six months (Table 2). Five control rabbits,

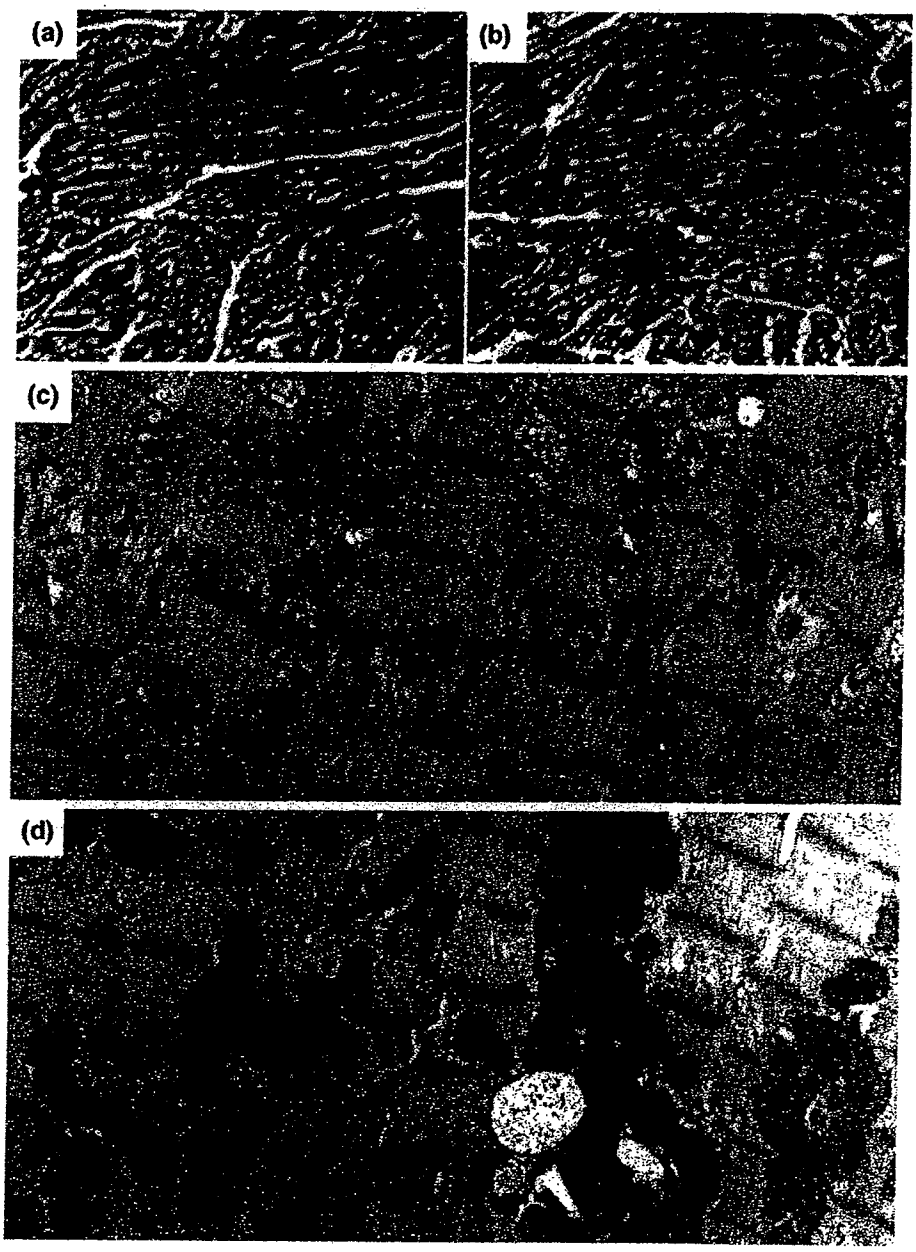


Figure 5 (a) Five μM section of control rabbit left ventricular free wall with trichrome stain at 200× magnification taken from a 6-month animal. Bar: 0.5 mm. (b) Comparable field in a line 66 mutant rabbit. (c) Electron micrograph of control rabbit left ventricular posterior papillary muscle. Bar: 0.77 μM. (d) Comparable section from a line 66 mutant rabbit.

7.5–14.5 months, four animals from line 4, two animals from line 11 and two animals from line 66 were used. There were 2 deaths temporally related to the procedure. One line 4 TG died from presumed air embolism into a coronary artery during catheter positioning. In another case, we were unable to obtain arterial access in a NTG animal. He recovered from anesthesia uneventfully and exhibited normal activity the following day but was subsequently found with a fractured vertebral column two days after the attempted catheterization. This animal was euthanized immediately and the heart tissue taken for RNA and protein analyses. There were no significant differences among the four groups in LV systolic pressure, LV end diastolic pressure, aortic systolic pressure or aortic diastolic pressure. Interestingly, every rabbit had a measurable pressure gradient between the LV and ascending aorta (ΔLV-AAo). Since LV obstruction was not detected by Doppler echocardiography in any experimental

Table 1 Echocardiographic data

	IVS	PW	LVEDD	LVESD	SF	HR	APM	PPM
Control ($n=7$)	2.7±5	2.0±4	15.9±2.6	10.2±2.2	35.4±8	256±26	0.6±0.0	0.63±0.1
Line 4 ($n=4$)	2.9±1	1.4±8	14.5±1.4	7.6±3.1	47.5±19.9	233±53	0.55±0.1	0.58±.05
Line 11 ($n=2$)	2.8±4	1.6±3	16.2±0.7	11.3±0.1	30±4.2	229±25	0.70±0.0	0.75±.07
Line 66 ($n=3$)	2.8±5	1.7±4	16.8±1.0	10.6±1.2	37.3±3.5	212±21	0.67±.01	0.67±.15

There were no significant differences in any parameter in any transgenic line *vs* control. IVS = interventricular septal thickness (mm), PW = posterior wall thickness (mm), LVEDD = left ventricular end-diastolic dimension (mm), LVESD = left ventricular end-systolic dimension (mm), SF = shortening fraction ([LVEDD-LVESD]/LVEDD), HR = heart rate (beats per minute), APM = anterior papillary muscle diameter (mm), PPM = posterior papillary muscle diameter (mm).

Table 2 Cardiac catheterization data

	LVSP	LVEDP	AAoP	ΔLV-AAo
Control ($n=3$)	100±7	10±4	85±5	15±3
Line 4 ($n=3$)	109±15	16±5	98±16	11±2
Line 11 ($n=2$)	104±25	14±2	84±17	2±6
Line 66 ($n=2$)	98±11	12±1	81±7	17±3

All values are mean ± SD. There were no significant differences in any parameter measured. LVSP = left ventricular systolic pressure (mmHg), LVEDP = left ventricular end diastolic pressure (mmHg), AaoP = ascending aortic systolic pressure (mmHg), ΔLV-AAo = left ventricular-ascending aorta pressure difference (mmHg).

Figure 6 Still frames from cineangiography of rabbit left ventricle. Images were obtained with the camera in the left axial oblique projection. The interventricular septum is profiled. (a) NTG and (b) line 66 rabbit hearts.

group, the etiology of the measurable gradient at catheterization might be due to partial obstruction of a relatively small outflow tract by the catheter. The small size of the rabbit heart precluded measuring accurate pullback pressures from LV apex → LV outflow tract (subaortic region) → AAo, but we found no angiographic evidence of mid-cavitary obstruction or overt papillary muscle hypertrophy in any rabbit studied [Figs 6(a), (b)]. Contractility was qualitatively normal by contrast angiography and there were no significant differences in $+dP/dt$ or $-dP/dt$ (data not shown).

Discussion

While the mouse remains the most widely used species in transgenic investigations, the rabbit offers an experimental model with significant advantages for cardiovascular research. Compared to the mouse, the larger size and slower heart rate of the rabbit is advantageous for physiological analyses such as echocardiography and cardiac catheterization. The rabbit heart also has a biochemical advantage. As in human hearts, the "slow" β-MHC isoform is the predominant heavy chain expressed in mature rabbits. In both species, ELC1a is expressed in the fetal ventricle with a switch to ELC1v after birth.[19,20] ELC1v has been detected in atria and ELC1a in ventricle of overloaded human heart, although this switch has not been documented in overloaded rabbit heart.[19,21] Relative to the mouse, rabbit and human cardiomyocytes have an increased contribution from the sarcolemmal Na^+/Ca^{2+} exchanger for cytosolic Ca^{2+} removal, with a lesser contribution from the SR-Ca^{2+}ATPase pump. In contrast, the smaller rodent heart relies more heavily on SR-Ca^{2+}ATPase as the primary mode of calcium removal.[22] Therefore, in terms of modeling human cardiac disease, the rabbit heart offers some unique opportunities.

Direct comparisons of mouse and rabbit phenotypes can be made from data obtained in studying the human β-MHC FHC mutation R403Q.[3,18] Using gene targeting, mice were made carrying the corresponding mutation in α-MHC (since α-MHC is the predominant isoform in the mouse ventricle). Mice homozygous for the mutation die in the first week of life, and while heterozygotes demonstrate some of the features of FHC, the mouse phenotype diverges from human disease in several critical

aspects including a relatively small increase in ventricular mass and a more severe phenotype in males, findings not associated with human FHC due to the R403Q mutation.[3,23,24] Using the mouse β-MHC promoter, β-MHC R403Q was expressed in transgenic rabbits[18] enabling the mutation to be placed in the endogenous β-MHC isoform. Transgenic rabbits with 41% replacement of endogenous β-MHC with the mutant protein showed a cardiac phenotype very similar to human R403Q disease, namely increased cardiac mass, histological evidence of myocyte disarray and interstitial fibrosis, and premature death. This model promises to be a valuable resource in the study of FHC and is a prime example of the importance of larger animal models of human cardiac disease since mice expressing the R403Q mutation in the endogenous α-MHC isoform do not completely recapitulate the human phenotype resulting from R403Q on an endogenous β-MyHC background, as is the case in rabbits and humans.[25]

We decided to model the human M149V ELC1v mutation in the rabbit. Using the mouse β-MHC promoter, we achieved 41% replacement of endogenous ELC1v with M154V early in life when the heart undergoes significant hypertrophy as rabbit body size increases rapidly. It must be noted that 41% replacement of endogenous ELC1v with a species-specific mutant may not be comparable to the 41% replacement of endogenous β-MHC with a human transgene achieved by Marian *et al.*[18] Despite rigorous investigation of this model in fully mature adults, we could not find any evidence of cardiac hypertrophy or any significant cardiac pathology even in animals older than 1 year. One possible reason for the lack of phenotype is the relatively small cohorts available for study, which could cause a type II statistical error. It is possible that with larger experimental groups, a subtle phenotype may have been detected. However, we found nothing that even remotely resembled the dramatic mid-cavitary obstruction phenotype described in humans with the M149V mutation. Additionally, we might find a phenotype if we examined older animals. Since rabbits achieve sexual maturity at 5 months and generally survive 5–6 years, the rabbits we studied would be considered "young adults". Because it is formally possible that a phenotype might present if we were to examine a geriatric cohort, we are aging a cohort of Line 66 TG rabbits for analysis later in life. However, if disease were likely to develop, we would expect to find some of the hypertrophic markers activated in the 12–15 month animals, as these markers present well before any overt pathology becomes apparent.[13] Also, we would expect to find some evidence of myofibril dysfunction as the basic substrate for eventual hypertrophy. We examined skinned fiber kinetics and mechanics to assess for any detectable alterations in myofibril function at a young age (5 weeks) when cardiac maturation is ongoing and transgenic replacement is significant (41%). While there was a very subtle increase in calcium sensitivity in TG compared to NTG littermates, there was certainly no evidence that the M154V mutation induced global changes in myofibril function that could culminate in cardiac dysfunction or hypertrophy.

The results are consistent with data derived from transgenic expression of the comparable mutation in the mouse ELC1v cDNA, where cohorts were maintained until they died of old age.[9] Interestingly, the hearts of these animals had decreased left ventricular mass, rather than the expected hypertrophic response. The results of single motor experiments performed in the mutant mice also contradicted those derived from experiments on human cardiac biopsy material from patients carrying the M149V mutation. In vitro motility assays performed with mutant human ventricular myosin showed increased actin translocation,[8] but in mutant mouse tissue this parameter was unaffected.[9] Reconciliation of these data sets with the data published by Vemuri *et al.* is problematic for several reasons, the most obvious being Sanbe's use of species specific mutant cDNA, as opposed to xeno-expression of a mutant genomic locus.[10] Regardless, in both the TG rabbits and mice that we have analyzed, the isolated M149V mutation is not sufficient to reproduce the human phenotype within the context of the endogenous ELC1v protein species.

This raises the broader question of how and when one assigns disease causality to an identified genetic defect. Koch's postulates, initially applied to the pathogenesis of infectious disease, are certainly worth consideration. In this case, the proposed causative agent is a mutation in the genomic sequence encoding ELC1v. This agent was isolated from an affected human host, and when delivered in the genomic form to a mouse, produced cardiac pathology. However, in two separate experimental models (FVBN mice and New Zealand White rabbits), the mutated coding sequence for ELC1v did not cause hypertrophic cardiomyopathy, indicating that the mutation cannot independently cause the FHC phenotype unless one invokes a specific interaction of the mutated amino acid only within the context of the endogenous human sequence. This remains a formal possibility since both our mouse and rabbit TG studies used

species-specific clones to avoid the confounding factor of functional differences across species. It is possible that strain differences could be responsible for the disparate phenotypes between the two mouse models, with C57B/J6 hybrid mice perhaps being more susceptible to hypertrophy than FVBN mice. Unexpected protein products arising from the human genomic sequence are yet another possibility. In our experiments, the potential for aberrant splicing events in heterologous genomic sequences was decreased by the use of cDNA clones. The data are therefore consistent with the hypothesis that the genesis of cardiac hypertrophy in patients carrying the M149V mutation is a more complex phenomenon than just expression of an altered sarcomeric protein. Clues to the unusual form of mid-cavitary obstruction and papillary muscle hypertrophy seen in patients carrying the M149V mutation perhaps might be found in equally unusual skeletal muscle pathology reminiscent of primary mitochondrial, or "ragged red fiber" disease.

Acknowledgements

This work was supported by NIH grants HL56370, HL60546, HL61638, HL52318 (to JR) and HL0379 (to JJ).

References

1. James J, Robbins J. Molecular remodeling of cardiac contractile function. *Am J Physiol* 1997; 273: H2105–2118.
2. Schiaffino S, Reggiani C. Molecular diversity of myofibrillar proteins: Gene regulation and functional significance. *Physiol Rev* 1996; 76: 371–423.
3. Geisterfer-Lowrance AA, Christe M, Conner DA, Ingwall JS, Schoen FJ, Seidman CE, Seidman JG. A mouse model of familial hypertrophic cardiomyopathy. *Science* 1996; 272: 731–734.
4. Tardiff JC, Factor SM, Tompkins BD, Hewett TE, Palmer BM, Moore RL, Schwartz S, Robbins J, Leinwand LA. A truncated cardiac troponin t molecule in transgenic mice suggests multiple cellular mechanisms for familial hypertrophic cardiomyopathy. *J Clin Invest* 1998; 101: 2800–2811.
5. Yang Q, Sanbe A, Osinska H, Hewett TE, Klevitsky R, Robbins J. A mouse model of myosin binding protein c human familial hypertrophic cardiomyopathy. *J Clin Invest* 1998; 102: 1292–1300.
6. Seidman C, Seidman J. Molecular genetics of inherited cardiomyopathies. In: Chien K (ed), *The Molecular Basis of Cardiovascular Disease: A Companion to Brunwald's Heart Disease*. Philadelphia, PA: WB Saunders Co Inc; 1999. pp. 251–263.
7. Lee WH, Hwang TH, Kimura A, Park SW, Satoh M, Nishi H, Harada H, Toyama J, Park JE. Different expressivity of a ventricular essential myosin light chain gene ala57gly mutation in familial hypertrophic cardiomyopathy. *Am Heart J* 2001; 141: 184–189.
8. Poetter K, Jiang H, Hassanzadeh S, Master SR, Chang A, Dalakas MC, Rayment I, Sellers JR, Fananapazir L, Epstein ND. Mutations in either the essential or regulatory light chains of myosin are associated with a rare myopathy in human heart and skeletal muscle. *Nat Genet* 1996; 13: 63–69.
9. Sanbe A, Nelson D, Gulick J, Setser E, Osinska H, Wang X, Hewett TE, Klevitsky R, Hayes E, Warshaw DM, Robbins J. *In vivo* analysis of an essential myosin light chain mutation linked to familial hypertrophic cardiomyopathy. *Circ Res* 2000; 87: 296–302.
10. Vemuri R, Lankford EB, Poetter K, Hassanzadeh S, Takeda K, Yu ZX, Ferrans VJ, Epstein ND. The stretch-activation response may be critical to the proper functioning of the mammalian heart. *Proc Natl Acad Sci USA* 1999; 96: 1048–1053.
11. Soldati T, Perriard JC. Intracompartmental sorting of essential myosin light chains: Molecular dissection and *in vivo* monitoring by epitope tagging. *Cell* 1991; 66: 277–289.
12. Schiller NB, Shah PM, Crawford M, DeMaria A, Devereux R, Feigenbaum H, Gutgesell H, Reichek N, Sahn D, Schnittger I, *et al.* Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. American society of echocardiography committee on standards, subcommittee on quantitation of two-dimensional echocardiograms. *J Am Soc Echocardiogr* 1989; 2: 358–367.
13. James J, Zhang Y, Osinska H, Sanbe A, Klevitsky R, Hewett TE, Robbins J. Transgenic modeling of a cardiac troponin i mutation linked to familial hypertrophic cardiomyopathy. *Circ Res* 2000; 87: 805–811.
14. James J, Osinska H, Hewett TE, Kimball T, Klevitsky R, Witt S, Hall DG, Gulick J, Robbins J. Transgenic over-expression of a motor protein at high levels results in severe cardiac pathology. *Transgenic Res* 1999; 8: 9–22.
15. Morano I, Hadicke K, Haase H, Bohm M, Erdmann E, Schaub MC. Changes in essential myosin light chain isoform expression provide a molecular basis for isometric force regulation in the failing human heart. *J Mol Cell Cardiol* 1997; 29: 1177–1187.
16. Fewell JG, Hewett TE, Sanbe A, Klevitsky R, Hayes E, Warshaw D, Maughan D, Robbins J. Functional significance of cardiac myosin essential light chain isoform switching in transgenic mice. *J Clin Invest* 1998; 101: 2630–2639.
17. James J, Sanbe A, Yager K, Martin L, Klevitsky R, Robbins J. Genetic manipulation of the rabbit heart via transgenesis. *Circulation* 2000; 101: 1715–1721.
18. Marian AJ, Wu Y, Lim DS, McCluggage M, Youker K, Yu QT, Brugada R, DeMayo F, Quinones M, Roberts R. A transgenic rabbit model for human hypertrophic cardiomyopathy. *J Clin Invest* 1999; 104: 1683–1692.
19. Sutsch G, Brunner UT, Von Schulthess C, Hirzel HO, Hess OM, Turina M, Krayenbuehl HP, Schaub MC. Hemodynamic performance and myosin light chain-1 expression of the hypertrophied left ventricle in aortic valve disease before and after valve replacement. *Circ Res* 1992; 70: 1035–1043.

20. SRIHARI T, TUCHSCHMID CR, SCHAUB MC. Isoforms of heavy and light chains of cardiac myosins from rat and rabbit. *Basic Res Cardiol* 1982; 77: 599–609.
21. PETERSON JN, NASSAR R, ANDERSON PA, ALPERT NR. Altered cross-bridge characteristics following haemodynamic overload in rabbit hearts expressing v3 myosin. *J Physiol* 2001; 536: 569–582.
22. BERS DM. Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res* 2000; 87: 275–281.
23. GEORGAKOPOULOS D, CHRISTE ME, GIEWAT M, SEIDMAN CM, SEIDMAN JG, KASS DA. The pathogenesis of familial hypertrophic cardiomyopathy: Early and evolving effects from an alpha-cardiac myosin heavy chain missense mutation. *Nat Med* 1999; 5: 327–330.
24. FATKIN D, MCCONNELL BK, MUDD JO, SEMSARIAN C, MOSKOWITZ IG, SCHOEN FJ, GIEWAT M, SEIDMAN CE, SEIDMAN JG. An abnormal Ca^{2+} response in mutant sarcomere protein-mediated familial hypertrophic cardiomyopathy. *J Clin Invest* 2000; 106: 1351–1359.
25. NAGUEH SF, KOPELEN HA, LIM DS, ZOGHBI WA, QUINONES MA, ROBERTS R, MARIAN AJ. Tissue doppler imaging consistently detects myocardial contraction and relaxation abnormalities, irrespective of cardiac hypertrophy, in a transgenic rabbit model of human hypertrophic cardiomyopathy. *Circulation* 2000; 102: 1346–1350.

EXHIBIT B

Clinical and Experimental Pharmacology and Physiology (1999) 26, 661–668

BRIEF REVIEW

GENE TRANSFER AND MODELS OF GENE THERAPY FOR THE MYOCARDIUM

M Yvonne Alexander,[†] Keith A Webster,[‡] Patricia H McDonald*[†] and Howard M Prentice*

**Division of Molecular Genetics, Institute of Biomedical and Life Sciences and [†]Department of Medicine and Therapeutics, University of Glasgow, Glasgow, UK, and [‡]Department of Molecular and Cellular Pharmacology, University of Miami Medical Center, Miami, Florida, USA*

SUMMARY

1. Gene transfer into the myocardium can be achieved through direct injection of plasmid DNA or through the delivery of viral vectors, either directly or through the coronary vasculature. Direct DNA injection has proven extremely valuable in studies aimed at characterizing the activities of promoter elements in cardiac tissue and for examining the influence of the pathophysiological state of the myocardium on expression of transferred foreign genes.

2. Viral vectors, in particular adenoviruses and adeno-associated virus, are capable of transfecting genetic material with high transduction efficiencies and have been applied to a range of model systems for *in vivo* gene transfer. Efficient gene transfer has been achieved into the coronary vessels and surrounding myocardium by intracoronary infusion of adenovirus.

3. Because the immunogenicity of viral vectors can limit transgene expression, much attention has been paid to strategies for circumventing this, including the development of new modified adenovirus and adeno-associated virus vectors that do not elicit significant inflammatory responses. While cellular transplantation may prove valuable for the repair of myocardial tissue, confirmation of its value awaits establishment of a functional improvement in the myocardium following cell grafting.

4. Because gene transfer into the myocardium can now be achieved with high efficiency in the absence of significant inflammatory responses, the ability to regulate foreign gene expression in response to an endogenous disease phenotype will enable the development of new effective viral vectors with direct clinical applicability for specified therapeutic targets.

Key words: adenovirus, animal models of foreign gene delivery, gene therapy, gene transfer, myocardial ischaemia, myocardium, viral vectors.

INTRODUCTION

Analyses of gene expression and promoter function in the intact heart are now possible through the advent of direct injection techniques to efficiently deliver plasmid and viral DNA into the myocardium. These techniques not only allow molecular genetic analyses of gene expression under different developmental and diseased states, but have also opened up new possibilities for gene therapy. Gene transfer into the heart and coronary vasculature by direct injection of naked, emulsified or encapsulated DNA is likely to have clinical as well as research applications in the very near future.

Injection of plasmid DNA, naked or combined with various mixtures of cationic lipids, has resulted in detectable levels of foreign gene expression *in vivo* in both skeletal and cardiac muscle. Viral vectors, in particular adenovirus (Ad) and adeno-associated virus (AAV), transfect genetic material with even higher transduction efficiencies and there is some debate about which transfer system is the best.[1–4] We will give an account of the recent advances of gene transfer using the different viral and non-viral methods, illustrating the important features of each approach. The strengths and weaknesses of the current vector systems will be reviewed and we will examine how vectors could be optimized for long-term gene expression. Finally, we will examine the potential for efficient delivery of these vectors in the clinical setting by intravenous injection and catheter-based techniques.

GENE TRANSFER IN THE MYOCARDIUM: DIRECT DNA INJECTION

Studies on the regulation of gene expression in the myocardium have relied heavily on transfection into primary cells, in particular neonatal cardiac myocytes and, to a more limited extent, adult myocytes. There are still no well-defined cardiac cell lines, although Simian virus 40 (SV40) large T-transformed atrial cells can be passaged and retain a cardiac phenotype.[5] Adult myocytes are difficult to isolate and maintain in culture for gene expression studies; they are also difficult to transfect and tend to dedifferentiate in long-term culture. While a wealth of information has been obtained using rat neonatal cardiac myocytes, these systems have been criticized for being *in vitro* as well as neonatal and rodent and, therefore, not an appropriate model for the analysis of heart function, neither diseased nor developmental. Although the neonatal rat cardiac myocyte

Correspondence: Howard M Prentice, Division of Molecular Genetics, Institute of Biomedical and Life Sciences, Anderson College, University of Glasgow, 56 Dumbarton Road, Glasgow G11 6NU, Scotland, UK. E-mail: <gbga91@udcf.gla.ac.uk>

Received 3 December 1998; revision 9 April 1999; accepted 14 April 1999.

List of abbreviations:

AAV	Adeno-associated virus	Luc	Luciferase
Ad	Adenovirus	α-MHC	α-Myosin heavy chain
β-Gal	Beta galactosidase	MuLV	Murine leukaemia virus
cTnC	Cardiac troponin C	p.f.u.	Plaque-forming unit
HRE	Hypoxia response element	PTGT	Percutaneous transluminal gene transfer
HSP	Heat-shock protein	SV40	Simian virus 40
HSV	Herpes simplex virus	Tet	Tetracycline
HVJ	Haemagglutinin virus of Japan	TGF-β	Transforming growth factor-β.
kb	Kilobase	VEGF	Vascular endothelial growth factor
LTR	Long-terminal repeat.		

models have generated important information on the regulation of gene expression in cardiac cells, as well as implications for disease responses, these results need to be validated in different species and in the context of the whole intact heart (*in vivo*). Cultures of human cardiac myocytes are likely to be important in this respect[6] and the recent explosion in transgenic mouse studies has certainly featured prominently and is likely to continue to do so in the future. In our opinion, combinations of the three systems (virus delivery *in vitro*, transgenic and direct injection) will most efficiently generate a complete molecular and cellular picture of heart disease and development. Direct transfer of DNA into cardiac muscle or vasculature currently has advantages over transgenic experiments in being cheaper, applicable to large as well as small animals and applicable to a wide range of developmental as well as disease models. Also, direct transfer of DNA into the adult cardiovascular system is likely to precede germ-line manipulations in the context of gene therapy.

In vivo transfection has been especially attractive for the myocardium, in terms of the information that could be determined about promoter activation in the whole heart responding to physiological (and pathophysiological) function under the influences of naturally occurring mechanical and hormonal stimuli. It was first shown by Wolff *et al.*[7] that injection of naked plasmid DNA directly into skeletal muscle of mice permitted reproducible expression and high fidelity of the foreign gene's promoter function. Subsequently, it was demonstrated that promoter chimeras injected into the heart responded appropriately to physiological hormonal stimuli[8] and, furthermore, that *cis*-acting elements may act differently *in vivo* compared with cell culture models.[9] The direct injection methodology combines some of the advantages of the *in vitro* transfection approach and of the transgenic mouse models in its application to short-term analysis of gene regulation.

Direct DNA injection has been especially valuable for studies directed at characterizing the activities of promoter elements in cardiac tissue and for examining transcriptional responses to physiological stimuli.[10] It was first demonstrated by Kitsis *et al.* that promoter elements transfected into the heart by direct DNA injection were capable of tissue-specific regulation and could respond appropriately to alterations in thyroid hormone levels *in vivo*.[8] The technique of direct DNA injection has also been applied to the characterization of the heart-specific activation properties of a 156 b.p. promoter region located immediately 5′ to the transcriptional start site of the slow/cardiac troponin C (*cTnC*) gene.[11] Other cardiac-specific promoter domains for the gene for the M isozyme of creatine kinase and for a ventricular-specific myosin light chain have been similarly characterized by direct injection of plasmid constructs. The method of direct injection may also be useful for investigating the effects of specific pathophysiological states on cardiac gene expression, such as ischaemia, hypertension and pressure or volume overload.[10,12,13]

Many groups have demonstrated the expression of naked plasmid DNA in the rat heart.[8,12,14–16] Our laboratory was the first to show that the pathophysiological state of the myocardium *in vivo* could dramatically influence the expression of transferred foreign genes[10,17] (and *vide infra*). There have been some discrepancies concerning reported stability of plasmid-based transgenes in both skeletal and cardiac muscle, but it seems likely that long-term expression of 3–4 months is quite feasible.[18] A gradual decline of transgene expression may be expected due to the episomal localization of the DNA in post-mitotic cardiac and skeletal muscle cells.[14,18]

The capacity to take up and express plasmid DNA following direct injection appears to be quite unique for striated muscle and does not occur in other organs at significant levels. The reasons for this are not clear, but it has been suggested that the cell damage and inflammation caused by the injection needle could mediate gene transfer by causing membrane disruption.[19] From electron microscope studies on injected skeletal muscle, Wolff *et al.*[20] demonstrated that colloidal gold, conjugated to plasmid DNA, crossed the external lamina and entered T tubules and caveolae, while gold conjugated with polylysine, polyethylene glycol or polyglutamate remained outside the fibres. These data suggest that DNA entry does not occur through transient membrane disruptions and does not appear to result from endocytosis. Wolff *et al.* suggest an alternative mechanism for DNA uptake by some type of cell membrane transporter, in particular via photocytosis. Microinjection of plasmid DNA into the cytoplasm of primary rat myotubes resulted in DNA entry into post-mitotic nuclei through the nuclear pore by a process common to other large karyophilic macromolecules.[21] This understanding of plasmid DNA nuclear entry may provide a basis for increasing the efficiency of direct plasmid gene transfer into heart and skeletal muscle. Directly injected plasmid DNA results in higher transfection efficiencies in the myocardium than in skeletal muscle when both tissues are injected with identical plasmid DNA in the same manner.[8] While the reason for this is unclear, it is possible that differences in structure of the T tubule system between skeletal and cardiac muscle may contribute to the discrepancies in transfection efficiency.[20]

Direct injection of naked plasmid DNA into the myocardium has only limited potential as a method for cardiac gene therapy because of the small number of cells per injection that express the transgene. Expression, as determined by histochemistry, appears to be localized around the site of injection with as few as 100–200 cells expressing the gene product.[14] Several investigators have studied the

parameters affecting gene expression following direct DNA injection. Increasing the quantity of DNA used for myocardial transfection did not alter levels of foreign gene expression,[22] whereas increasing the volume of injectate, while maintaining a constant quantity of DNA (50 μg in this case) appeared to increase expression from a luciferase-encoding plasmid. It has been further demonstrated that transfection efficiencies are higher with closed circularized DNA than with linearized DNA,[14] but the total number of expressing cells remains small and the technique is probably only suitable for secreted gene products.

A critical requirement for gene therapy protocols is the ability to achieve control of the foreign gene expression appropriately in the host. Fishman *et al.*[23] have developed a tetracyline (tet)-regulated gene expression system, based on injection into adult rat hearts of a Tet-repressor VP16 transactivator plasmid with a luciferase (luc) target gene. Using this system it was possible to induce luciferase expression by two orders of magnitude in response to small changes in input-controlled transactivator DNA, allowing target gene expression to be induced or repressed by altered antibiotic administrations.

Our own studies have demonstrated that specific promoter elements can be used to regulate transgene expression in response to physiological stress.[10,17] In our experiments, we examined the degree of regulation of hypoxia-responsive promoter constructs in an experimental model of myocardial ischaemia with reperfusion. Hypoxia is a critical and obligatory component of ischaemic tissue and solid tumours, where the hypoxic zone is restricted to the diseased tissue. It should be possible to tightly regulate transgene expression in these diseased tissues through the use of hypoxia as an endogenous regulator. To investigate transgene regulation in myocardial ischaemia, we constructed an expression plasmid containing multiple copies of a hypoxia response element (HRE) from the erythropoetin gene placed upstream of a minimal α-myosin heavy chain (α-MHC) promoter driving a luciferase reporter. In cell culture, expression of this test promoter (α-MHC86HRE) was highly muscle specific and was induced approximately 10-fold by hypoxia. In a rabbit model of 15 min myocardial ischaemia followed by reperfusion, expression of the construct was induced by four- to five-fold at 1 and 4 h post-ischaemia, returning to basal levels by 8 h.[10] These results show, for the first time, that it is possible to tightly regulate a transgene plasmid or virus vector in response to the disease phenotype and this represents a significant milestone in gene therapy research.

VECTORS FOR MYOCARDIAL GENE TRANSFER

Retroviruses

The retrovirus is a small RNA virus, packaged within a glycoprotein envelope. The life cycle of the retrovirus is now well characterized.[24] Following infection into its host, the viral RNA is reverse-transcribed into a double-stranded DNA molecule that then becomes integrated into the cellular genome. By exploitation of this stable integration into the host genome following infection, it was anticipated that long-term expression of the recombinant gene would be achieved. However, as an unexplained loss of exogenous gene expression is frequent with retroviral vectors and integration of retroviral sequences is dependent on cell division,[25] they may prove to be of little use for the myocardium. Nevertheless, initial experiments with retroviral vectors carrying the β-galactosidase (β-*gal*) gene showed promising results using *ex vivo* methods in heart and skeletal muscle; Salvatori *et al.* achieved 50% gene transfer efficiency in foetal myoblasts and adult satellite cells, which in turn led to expression in muscle fibres following *in vivo* transplantation.[26] A similar retrovirus carrying the β-*gal* reporter gene was used for infection of murine foetal cardiac myocytes in culture, which were then transplanted into syngeneic adult mice.[27] Histological analysis of the grafted hearts indicated expression of the β-gal gene product in the transplanted cells for as long as 6 months and revealed an absence of inflammation or scar tissue over this time period.[27] Clinical use of retroviruses as gene therapy vehicles may be limited by the potential for oncogenicity and insertional mutagenesis. Retroviral protocols are also limited by the relatively low viral titres that can be obtained and by the limited size of foreign sequence they can accommodate.[28]

Adenoviruses

Replication-deficient adenovirus (Ad) are currently among the most efficient vectors for transferring genes to a wide variety of cell types *in vivo*.[2,3] In a comparison of gene transfer using plasmid DNA and recombinant Ad DNA, it was found that the efficiency of transduction with Ad was at least an order of magnitude higher than that observed with plasmid DNA, tissue penetration was vastly improved and transgene expression was proportionately increased.[29] Unique properties of adenoviral vectors include an exceptionally high efficiency of infection and the ability to accommodate large fragments of foreign DNA (up to 8 kb). Of particular importance for gene transfer to the myocardium is the property to efficiently infect both proliferating and terminally differentiated cells. In contrast with retroviruses, Ad can be prepared at much higher stock concentrations than retroviruses, with typical titres of 10^{11} plaque-forming units (p.f.u.)/mL, 10 000-fold higher than equivalent retroviral titres. Furthermore, the adenoviral genome remains episomal, so that the potential for oncogenesis and insertional mutagenesis is avoided.

As with all current vectors, there are limitations to the use of the Ad. The first-generation of Ad5 vectors has most of the Ad genome and expresses multiple Ad proteins, including the penton protein, which is associated with cytotoxicity, particularly at a high multiplicity of infection.[30] Virus capsid proteins also stimulate a humoral response, activating neutralizing antibodies, inflammation and elimination of both Ad vector and host cell.[31] Second- and third-generation Ad vectors, in which a region of 28 kb spanning all adenoviral coding sequences has been deleted, have recently been developed to circumvent the inflammatory responses associated with Ad infection. Such vectors, which require helper viruses for their generation, can accommodate large DNA fragments encoding multiple foreign genes.[32]

Adeno-associated viral vectors

Because of the inflammatory responses associated with infection by Ad and other viral vectors, attention has been paid to the potential for using viruses that are defective in that they express no viral genes. Adeno-associated virus is an example of such a defective virus, devoid of viral genes but capable of permitting foreign genes to be packaged into a viral coat.[4,33] This non-pathogenic human parvovirus differs from Ad in that it generally integrates into a specific site in the genome of the host cell and may provide for longer-term

transgene expression, although producing sufficiently high titres remains a problem.[1-34] Direct injection of AAV into rat hearts resulted in transgene expression 2 months after administration of the virus, with no detectable inflammatory response.[35] Delivery of an AAV vector into the coronary vasculature of pigs by percutaneous intra-arterial infusion using a routine catheter technique resulted in expression in cardiac myocytes for at least 6 months after injection, without toxicity or inflammation.[33,35-43] Because of its high infectivity and low immunogenicity, AAV gene transfer shows potential as a highly effective gene transfer vector for myocardium, in particular as methods become available for increasing the yield of virus[44] and for increasing transduction efficiencies by infecting in the presence of Ad gene products.[42]

Herpes simplex virus

The ability of Herpes simplex virus (HSV1) vectors to accommodate very large foreign DNA fragments has rendered them attractive as a gene transfer system with potential advantages over other viral systems. While HSV1 has been proposed as a candidate gene therapy vector for the nervous system because of its ability to latently infect neurons, it is also capable of infecting a range of other cell types where the vector may prove valuable if lytic infection can be inhibited. Coffin *et al.* achieved successful gene transfer in cardiac myocytes and vascular smooth muscle cells *in vitro* using three different disabled HSV vectors, with an efficiency of transfection of the vascular smooth muscle cells that was less than that observed with myocytes. In the rat heart *in vivo*, successful transfection was obtained with no apparent change in cellular morphology, suggesting negligible cytopathic effects. Mesri *et al.* used a replication-defective HSV1 vector carrying the gene encoding the angiogenic factor vascular endothelial growth factor (VEGF) to infect fibroblasts *in vitro* for subsequent transplantation studies. When the infected fibroblasts were injected into syngeneic mice, transgene expression was found to be capable of eliciting an angiogenic response.[45] This study pointed to the possibility that a HSV1 vector could have potential for applications for inducing focal angiogenesis to ameliorate myocardial ischaemia. Because HSV1 can transfer large DNA fragments, such gene therapy vectors may be particularly applicable to myocardial disorders requiring large genomic substitutions or the use of extensive genetic control regions.

Haemagglutin virus of Japan

The haemagglutin virus of Japan (HVJ) is an inactivated paramyxovirus that has been used within a liposome complex that entraps DNA. In transfections of cardiac myocytes in culture, it was shown that 90% transfection efficiency could be obtained and that foreign gene expression was detectable over 7 days.[46] Intracoronary injection of the HVJ/liposome complexes was also effective for providing efficient gene transfer into cardiac myocytes *in vivo*, with expression lasting for 1 week.[47] Directly injected HVJ/liposome DNA has been shown to result in higher reporter gene expression 3 days after administration than that obtained from injection of naked plasmid DNA.[46] Introduction of the HVJ/liposome complex containing a β-gal-encoding vector within the pericardium resulted in widespread staining of cardiac myocytes and fibroblasts. The HVJ-mediated gene transfer by direct infusion into the coronary artery resulted in staining of cardiac myocytes around the microvasculature.[46] *In vivo* intracoronary infusion of the HVJ/liposome complex containing the heat-shock protein 70 (*HSP70*) gene in rats resulted in enhanced tolerance of Langendorff perfused hearts to ischaemia–reperfusion injury.[48]

MODEL SYSTEMS FOR *IN VIVO* GENE TRANSFER

In vivo studies using small animal models

Several groups have assessed the specificity, efficiency and duration of Ad-mediated gene expression in the myocardium.[22,49-54] Stratford-Perricaudet *et al.*[49] were the first group to demonstrate long-term *in vivo* gene transfer throughout mouse skeletal and cardiac muscles after intravenous administration of a recombinant adenovirus (1×10^9 p.f.u./mL). It was demonstrated that approximately 0.2% of neonatal cardiomyocytes were transduced at 15 days after injection of the Ad. Reporter gene expression was found to persist, but decreased over a period of 12 months. In adult mice, intravenous Ad administration resulted in less efficient gene transfer.[49]

Guzman *et al.* used a sub-diaphragmatic approach for myocardial gene transfer[50] rather than the thoracic approach of previous studies[8,18] in order to avoid the high mortality associated with intubation and ventilation. In an analysis of myocardial gene transfer in the rat by sub-diaphragmatic injection of 5×10^8 p.f.u. Ad or 200 μg plasmid DNA,[50] it was demonstrated that Ad delivery was considerably more efficient than plasmid injection. Adenoviral injection resulted in significant foreign gene expression in cardiac myocytes, with maximal expression during the first week following injection, decreased transgene expression at 10–15 days after injection and no detectable foreign gene expression at 30 days. These investigators observed an acute inflammatory response in hearts injected with Ad. Because an inflammatory response has also been noted with plasmid DNA injection,[18,22] the authors suggest the inflammatory response may be related to injury produced by direct injection rather than to a stimulation of an immunological response to viral gene products.

In vivo studies using large animals: Relevance to humans

While many cardiac studies have been carried out in small animal models that may be informative in terms of cardiac function and ventricular performance under various inotropic and loading conditions,[55] it is important to use large animal models with physiology similar to that of a human and for consideration of potential applications to human gene therapy protocols. In a pig model, injection of replication-deficient recombinant Ad vectors carrying the *Luc* gene resulted in significant reporter gene activity detected at 3 days, increasing markedly at 7 days and then declining progressively at 14 and 21 days.[56] Comparable levels of foreign gene expression were found between plasmid and Ad injection but, when compared on a molar basis (i.e. when normalized to the number of genes injected), Ad-mediated gene transfer was found to be 140 000-fold more efficient than plasmid delivery. Injection of a β-gal-encoding Ad resulted in foreign gene expression that was localized predominantly to cardiac myocytes. The amount of recombinant protein expressed correlated closely with the quantity of virus injected in a 100 μL volume, over a range of virus concentrations ranging from 0.7×10^9 to 3.6×10^9 p.f.u./mL. No detrimental effects on ventricular function were found, although there was evidence of pronounced leucocytic infiltration in the virally infected myocardium.[56] In trying to circumvent the immune response, Kaplitt *et al.*[35] delivered an AAV

vector percutaneously by intra-arterial infusion into the coronary vasculature of a pig. Delivery of this vector by a routine catheter technique resulted in transgene expression in cardiac myocytes for at least 6 months after injection without toxicity or inflammation.[35]

DELIVERY METHODS

One of the key elements in successful gene transduction of the target cells is the method used in delivering the viral vector or plasmid DNA to the host cells. Initial attempts to programme recombinant gene expression in the myocardium were limited by low efficiencies of gene transfer and by the need for intramyocardial injections of DNA or Ad.[14,50,53] Barr *et al.*[57] described a more efficient method for gene transfer into both the coronary vessels and surrounding myocardium, using catheter-mediated gene transfer. Intracoronary infusions of Ad are relatively non-invasive and can be performed percutaneously using established cardiac catheterization techniques. Unlike direct injection into the myocardium, intracoronary infusion of Ad into the rabbit myocardium did not induce inflammation or myocardial necrosis.[57] As many as 32% of cardiac myocytes expressed the recombinant gene at 5 days following infusion, but only 0.01% of cells in four of seven animals expressed the gene at 1–2 months. However, levels of gene expression in the myocardium were 10–50-fold higher by comparison with those obtained by direct DNA injection. In a similar study, Li *et al.* examined the feasibility, efficiency and safety of Ad-mediated gene transfer *in vivo* into canine myocardium by percutaneous transluminal gene transfer (PTGT) using a needle catheter.[58] Injections into the left ventricle of dogs through a needle catheter inserted via a femoral artery were performed using either replication-deficient Ad or plasmid, both expressing β-gal. Expression of lacZ was examined by histochemical staining and quantified by measuring β-gal activity. Injection with 1.0×10^9 p.f.u. recombinant Ad induced lacZ expression at levels at least 10-fold higher than those obtained with a 50 μg injection of plasmid-expressing lacZ. Foreign β-gal expression was detected within 24 h, peaked at 7 days and persisted for 2 weeks after gene transfer. The duration and levels of gene expression coincide with the results reported by Barr *et al.*[57] A comparison of delivery methods in porcine heart indicated that intramyocardial injection of recombinant Ad was more efficient for short-term gene transfer than intracoronary infusion.[53] This observation was in contrast with data from intracoronary infusion of AAV using these standard catheterization techniques and demonstrating successful long-term gene transfer in the porcine model.[35] As an alternative to intravascular administration, Lamping *et al.*[59] have used an Ad encoding a nuclear-targeted β-gal to investigate vector delivery into the pericardial sac of dogs. One day after injection, transgene expression was observed in the parietal pericardium and left atrial tissue, with lower levels detectable in the right and left ventricles. Histochemical analysis indicated expression of the transgene in the visceral pericardium of atria and ventricles and, occasionally, in the epicardial myocytes, arterioles and venules.[59]

IMMUNOGENICITY AND NEW VECTOR DESIGN

Because a major barrier in obtaining efficient gene transfer has been the problem of transient foreign gene expression and an inflammatory response, it has become necessary to focus on the nature of the immune response and to consider ways of circumventing it. Studies aimed at identifying the immune effector involved in determining the longevity of virally delivered transgene expression have demonstrated that immunodeficient rats display higher levels and more prolonged durations of foreign gene expression than immunocompetent rats.[60] Ablation of CD4+ T cell activation at the time of vector administration has been used as a strategy for preventing cellular and humoral immunity.[61] By administration of immune-modulating agents, such as monoclonal antibodies to CD4+ cells and to CD40 ligand, it has been possible to prevent the effector response of CD8+ T and B cells.[61] A comparable immunosuppressive effect has been reported in studies using transforming growth factor (TGF)-β1 or vil10.[40]

An alternative strategy to immunomodulation is the development of novel viral vectors that may elicit decreased immune responses. Heavily deleted gutless Ad vectors that are depleted of all viral genes are capable of encoding multiple foreign genes and can retain their ability to infect target cell types.[62–64] An Ad dodecahedron made of Ad pentons or penton bases retains many of the properties of Ad, including efficiency of entry and efficient release of DNA from endosomes, but may have the advantage of being non-immunogenic.[64] These gutless Ad vectors and the AAV vectors currently hold the most promise for the future as vectors for delivery of therapeutic genes to the heart and vasculature.

CELLULAR TRANSPLANTATION

Myocardial regeneration and autologous grafting

Because the adult mammalian myocardium is incapable of regeneration, considerable attention has been directed towards replacing damaged myocardium with new viable muscle.[65] Cardiac myocytes transformed with SV40 large T antigen, which have been used in pilot experiments for grafting into hearts of syngeneic mice, were shown to fuse successfully with the host myocardium and were viable for as long as 4 months postimplantation.[66] The transformed nature of the transplanted cells from these studies led to concerns over their unregulated growth potential. The grafts were not viable in the long term and there was no evidence for improved performance of the myocardium.

In studies on the transplantation of skeletal myoblasts into ventricular myocardium, C2C12 skeletal myoblasts were shown to be capable of forming long-term differentiated grafts with evidence of intercalated discs and gap junctions with aligned cardiac cells and repressed myoblast proliferation in the hearts of syngeneic mice.[67] Again, there was evidence of tumorigenesis and no evidence for improved cardiac function. Allogeneic and xenogeneic myoblasts injected into the anterior and posterior walls of the porcine left ventricle were successfully transplanted in immunosuppressed animals with no significant graft rejection.[68] No reports have yet claimed to show changes in myocardial function in response to cellular transplantation.

Foetal cardiac myocytes have been successfully implanted into the hearts of adult mice with a demonstration of the presence of intercalated discs between the grafted cells and the host myocardium.[69] Following engraftment of foetal cardiomyocytes into the myocardium of dystrophic mice and dogs, there was clear morphological evidence of spontaneous fusion of host and donor cells.[70] Autologous transplantation of cardiac myocytes would be clearly preferable to avoid graft rejection, but the accessibility of donor cardiac myoblasts is likely to limit the clinical applicability unless there are breakthroughs in regeneration research. Cardiac myocytes differentiated

in culture from embryonic stem cells[71] may eventually provide a source of cells/tissue for *in vivo* grafting experiments.[40]

One means of achieving immunosuppression as well as delivering therapeutic genes has been to inject genetically engineered skeletal myoblasts into the myocardium or skeletal muscle where they secrete a foreign gene product.[72,73] Retrovirally transduced allografts, expressing TGF-β1 under the control of the SV40 promoter or interleukin (IL)-10 gene under the control of murine leukaemia virus long-term repeat (MuLV)-LTR, displayed increased survival consistent with an induced immunosuppression.[72]

Cellular transplantation into scar or infarct tissue

An alternative approach for myocardial repair may be to induce differentiation of skeletal muscle within cardiac muscle from transplanted satellite cells. Skeletal muscle satellite cells are undifferentiated myoblasts that remain dormant under the basal lamina but become activated upon myofibre injury, when they migrate towards the damaged site, enter a mitotic cycle and differentiate into skeletal muscle.[74] Satellite cells, when implanted into injured myocardium, can be influenced by the myocardial environment to display a cardiac myofibre-like phenotype.[75,76] Such cells implanted into a site of cryoinjury were found to differentiate into muscle fibres and showed evidence of intercalated discs.[76] Such satellite cells may withstand a short-term ischaemic episode[77] and could provide a valuable myocardial repair strategy. Foetal myocardial tissue has also been successfully transplanted into myocardial scar tissue with graft survival from 7 to 24 days after infarction: a duration of viability that may support delivery of a range of therapeutic proteins.[78] Implantation of neonatal skeletal myocytes into scar tissue resulted in the establishment of new muscle tissue that was capable of contracting when electrically stimulated.[79] At day 1, grafted cells were proliferating and did not express MHC and by 2 weeks the grafts began expressing β-MHC, a characteristic feature of slow skeletal myocytes. Co-expression of embryonic, fast and β-MHC continued over 3 months, consistent with conversion of the grafts to slow twitch fibres that could prove to be suited to a cardiac work load.

MOLECULAR CARDIOMYOPLASTY

Dynamic cardiomyoplasty is a therapeutic procedure that involves wrapping a synchronously paced skeletal muscle, usually the latissimus dorsi, around the heart.[80,81] A potentially analogous gene therapy approach to cardiomyoplasty would be to use genetic manipulation to regenerate cardiac myocytes after myocardial infarction. On overexpression, the myogenic determination factor MyoD[82,83] is capable of inducing skeletal muscle differentiation of a number of cell types, including fibroblasts. A number of recent studies, including our own, have addressed the possibility that forced expression of MyoD could be used for converting non-myocytes within infarcted or scarred myocardial tissue with a positive therapeutic outcome.[82,84,85] We have demonstrated that overexpression of MyoD through retroviral delivery into primary neonatal cardiac fibroblasts in culture will result in the formation of elongated multinucleated myotubes. Upon delivery of the retrovirus by injection into infarcted dog myocardium, we identified rare clusters of cells that stained positive for skeletal muscle-specific skeletal fast MHC.[84]

In healing rat hearts injured 1 week previously, it was found that cardiac granulation (wound repair) tissue could be successfully infected with a MyoD-encoding Ad. Infected cardiac granulation tissue expressed MyoD mRNA and there was evidence of structures suggesting multinucleated myotubes.[85]

SUMMARY/CONCLUDING REMARKS

There have been major advances in myocardial gene transfer in recent years, with successful demonstrations of high transfection efficiencies, high-level transgene expression and regulation of foreign gene expression in a manner that would be applicable to gene therapy for the diseased myocardium. The principal methods for foreign gene delivery to the myocardium have involved direct introduction of transgenes into cardiac myocytes and grafting of cardiac myocytes or myoblasts that have been transfected *ex vivo* with a foreign gene. Both direct gene transfer and cell grafting have limitations. Initial studies on direct gene transfer by plasmid or viral vectors resulted in poor transfection efficiencies and a lack of sustained transgene expression in cardiac myocytes. While direct plasmid gene transfer is inefficient, delivery of Ad-based vectors has resulted in inflammatory responses that elicit a shut-off of foreign gene expression. New modified Ad and AAV have been particularly effective for achieving high-level foreign gene expression in cardiac myocytes in the absence of a significant inflammatory reaction. Cellular transplantation may hold promise for repair of compromised myocardial tissue, but its application to gene therapy approaches must await the demonstration of a functional improvement in the myocardium following cell grafting.

A critical requirement for an effective gene therapy vector is the ability to regulate levels of gene expression temporally and spatially in a manner that is appropriate to the disease state. While this could be achieved through hormone or drug-responsive promoters, this would require additional monitoring and exogenous administration of an additional drug that would result in clinical procedures that do not significantly differ from those of conventional medicine. Our recent demonstration of hypoxia-responsive transgene expression in a model of myocardial ischaemia with reperfusion is the first demonstration of regulation of a transgene in response to an endogenous disease phenotype that is fundamental for the development of future successful gene therapy vectors.

As many of the initial concerns and limitations are becoming eliminated, it is clear that foreign genes can be delivered to the myocardium with high efficiency and that appropriate vectors are now available to ensure adequate gene transfer in the absence of significant inflammatory responses. The ability to regulate transgene expression appropriately for a particular disease phenotype will now permit development of new effective viral vectors for clinical applications directed at therapeutically relevant targets.

ACKNOWLEDGEMENTS

The authors' work in this area has been supported by grants from the National Institutes of Health (KAW), British Heart Foundation, Wellcome Trust, Medical Research Council, Royal Society (HMP), by the Cigarette and Tobacco Surtax of the State of California (KAW) and by a Wellcome Trust Collaborative Grant (KAW, HMP).

REFERENCES

1. Carter BJ. The promise of adeno-associated virus vectors. *Nature Biotechnol.* 1996; 14: 1725–6.

2. Ring CJA. Adenovirus vectors. In: Lemoine NR (ed.). *Gene Therapy*. BIOS Scientific Publishers, Oxford. 1996; 61–76.
3. Brody SL, Crystal RG, Boris-Lawrie K, Ledley FD, Curiel DT. Adenovirus-mediated *in vivo* gene transfer. *Ann. N.Y. Acad. Sci.* 1997; **716**: 90–103.
4. Flotte TR, Carter BJ. Adeno-associated virus vectors for gene therapy. *Gene Ther.* 1995; 2: 357–62.
5. Claycomb WC, Lanson NA, Stallworth BS *et al.* HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl Acad. Sci. USA* 1998; **95**: 2979–84.
6. Stave D. Kohtz. Studies of cardiomyocytes in culture: A developmental perspective. In: Marks AR, Taubman MB (eds). *Molecular Biology of Cardiovascular Disease*. Marcel Dekker, New York. 1997; 81–110.
7. Wolff JA, Malone RW, Williams P. Direct gene transfer into mouse muscle *in vivo*. *Science* 1990; **247**: 1465–8.
8. Kitsis R, Buttrick P, McNally E, Kaplan M, Leinwand L. Hormonal regulation of a gene injected into rat heart *in vivo*. *Proc. Natl Acad. Sci. USA* 1991; **88**: 4138–42.
9. Buttrick PM, Kaplan ML, Kitsis RN, Leinwand LA. Distinct behavior of cardiac myosin heavy chain gene constructs *in vivo*: Discordance with *in vitro* results. *Circ. Res.* 1993; **72**: 1211–17.
10. Prentice H, Bishopric NH, Hicks MN *et al.* Regulated expression of a foreign gene targeted to the ischaemic myocardium. *Cardiovasc. Res.* 1997; 35: 567–74.
11. Parmacek MS, Ip HS, Jung F *et al.* A novel myogenic regulatory circuit controls slow/cardiac troponin c gene transcription in skeletal muscle. *Mol. Cell. Biol.* 1994; 14: 1870–85.
12. Prentice H, Kloner RA, Li Y, Newman L, Kedes L. Ischemic/reperfused myocardium can express recombinant protein following direct DNA or retroviral injection. *J. Mol. Cell. Cardiol.* 1996; 28: 133–40.
13. McDonald P, Hicks MN, Cobbe SM, Prentice H. Gene transfer in models of myocardial ischemia. *Ann. N.Y. Acad. Sci.* 1995; **752**: 455–9.
14. Acsadi G, Jiao S, Jani A, Duke D. Direct gene transfer and expression into rat heart *in vivo*. *New Biol.* 1991; 3: 71–81.
15. Hasegawa K, Kitsis RN. Gene transfer into adult cardiac myocytes *in vivo* by direct injection of DNA. In: Marks AR, Taubman MB (eds). *Molecular Biology of Cardiovascular Disease*. Marcel Dekker, New York. 1997; 67–80.
16. Prentice H, Kloner RA, Prigozy T *et al.* Tissue restricted gene expression assayed by direct DNA injection into cardiac and skeletal muscle. *J. Mol. Cell. Cardiol.* 1994; 26: 1393–401.
17. Webster KA, Prentice H, Discher DJ, Hicks MC, Bishopric NH. Targetting and regulating the expression of foreign genes in ischemic tissue. In: Whelan WJ (ed.). *Molecular Biology in the Conquest of Disease*. Oxford University Press, Oxford. 1998; 37–38.
18. Lin H, Parmacek M, Morle G, Bolling S, Leiden J. Expression of recombinant genes in the myocardium *in vivo* after direct injection of DNA. *Circulation* 1990; 82: 22 117–21.
19. McNeil PL, Kahkee R. Disruptions of muscle fibre plasma membranes: Role in exercise-induced damage. *Am. J. Pathol.* 1992; **140**: 1097–109.
20. Wolff JA, Dowty ME, Jiao S *et al.* Expression of naked plasmids by cultured myotubes and entry of plasmids into t tubules and caveolae of mammalian skeletal muscle. *J. Cell Sci.* 1992; **103**: 1249–59.
21. Dowty ME, Williams P, Zhang G, Hagstrom JE, Wolff JA. Plasmid DNA entry into post-mitotic nuclei of primary rat myotubes. *Proc. Natl Acad. Sci. USA* 1995; **92**: 4572–6.
22. Gal D, Weir L, Leclerc G, Pickering JG, Hogan J, Isner JM. Direct myocardial transfection in two animal models: Evaluation of parameters affecting gene expression and percutaneous gene delivery. *Lab. Invest.* 1993; 68: 18–25.
23. Fishman GI, Kaplan ML, Buttrick PM. Tetracycline-regulated cardiac gene expression *in vivo*. *J. Clin. Invest.* 1994; **93**: 1864–8.
24. Gunzburg W, Salmons B. Retroviral vectors. In: Lemoine NR (ed.). *Gene Therapy*. BIOS Scientific Publishers Ltd, Oxford. 1996: 33–60.
25. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* 1990; **10**: 4239–42.
26. Salvatori G, Ferrari G, Mezzogiorno A *et al.* Retroviral vector-mediated gene transfer into human primary myogenic cells leads to expression in muscle fibers *in vivo*. *Hum. Gene Ther.* 1993; 4: 713–23.
27. Gojo S, Kitamura S, Germeraad WTV, Yoshida Y, Niwaya K, Kawachi K. *Ex vivo* gene transfer into myocardium using replication-defective retrovirus. *Cell Transplant.* 1996; 5: S81–4.
28. Vile RG, Russell SJ. Retroviruses as vectors. *Br. Med. Bull.* 1995; **51**: 12–30.
29. Kass-Eisler A, Falck-Pedersen E, Alvira M *et al.* Quantitative determination of adenovirus-mediated gene delivery to rat cardiac myocytes *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA* 1993; **90**: 11 498–502.
30. Wells KE, Maule J, Kingston R *et al.* Immune responses, not promoter inactivation, are responsible for decreased long-term expression following plasmid gene transfer into skeletal muscle. *FEBS Lett.* 1997; **407**: 164–8.
31. Gilgenkrantz H, Duboc D, Juillard V *et al.* Transient expression of genes transferred *in vivo* into heart using first-generation adenoviral vectors: Role of the immune response. *Hum. Gene Ther.* 1995; **6**: 1265–74.
32. Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT. A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc. Natl Acad. Sci. USA* 1996; 93: 5731–6.
33. Lynch CM, Hara PS, Leonard JC, Williams JK, Dean RH, Geary RL. Adeno-associated virus vectors for vascular gene delivery. *Circ. Res.* 1997; **80**: 497–505.
34. Berns KI, Giraud C. Biology of adeno-associated virus. *Curr. Topics Microbiol. Immunol.* 1996; 218: 1–23.
35. Kaplitt MG, Xiao X, Samulski RJ *et al.* Long-term gene transfer in porcine myocardium after coronary infusion of an adeno-associated virus vector. *Ann. Thoracic Surg.* 1996; **62**: 1669–76.
36. Clark KR, Sferra TJ, Johnson PR. Recombinant adeno-associated viral vectors mediate long-term transgene expression in muscle. *Hum. Gene Ther.* 1997; 8: 659–69.
37. Ponnazhagan S, Mukherjee P, Yoder MC *et al.* Adeno-associated virus 2-mediated gene transfer *in vivo*: Organ-tropism and expression of transduced sequences in mice. *Gene* 1997; **190**: 203–10.
38. Rolling F, Nong Z, Pisvin S, Collen D. Adeno-associated virus-mediated gene transfer into rat carotid arteries. *Gene Ther.* 1997; 4: 757–61.
39. Conrad CK, Allen SS, Afione SA *et al.* Safety of single-dose administration of an adeno-associated virus (aav)–cftr vector in the primate lung. *Gene Ther.* 1996; 3: 658–68.
40. Qin L, Chavin KD, Ding Y *et al.* Multiple vectors effectively achieve gene transfer in a murine cardiac transplantation model: Immunosuppression with TGF-beta1 or vil-10. *Transplantation* 1995; 59: 809–16.
41. Arnold TE, Gnatenko D, Bahou WF. *In vivo* gene transfer into rat arterial walls with novel adeno-associated virus vectors. *J. Vasc. Surg.* 1997; 25: 347–55.
42. Fisher KJ, Jooss K, Alston J *et al.* Recombinant adeno-associated virus for muscle directed gene therapy. *Nature Med.* 1997; 3: 306–12.
43. Samulski RJ, During MJ, Kaplitt MG, Xiao X, Redman G, McCown T. Adeno-associated virus vectors yield long-term expression and delivery of potentially therapeutic genes into non-dividing neuronal cells. *J. Neurovirol.* 1997; 3 (Suppl. 1): S72.
44. Maxwell F, Harrison GS, Maxwell IH. Improved production of recombinant AAV by transient transfection of nh324k cells using electroporation. *J. Virol. Methods* 1997; 63: 129–36.
45. Mesri EA, Federoff HJ, Brownlee M. Expression of vascular endothelial growth factor from a defective herpes simplex virus type 1 amplicon vector induces angiogenesis in mice. *Circ. Res.* 1995; 76: 161–7.
46. Aoki M, Morishita R, Muruishi A *et al.* Efficient *in vivo* gene transfer into the heart in the rat myocardial infarction model using the HVJ (hemagglutinating virus of Japan)–liposome method. *J. Mol. Cell. Cardiol.* 1997; 29: 949–59.

47. Weebster AS, Sawa Y, Kadoba K *et al.* Efficient gene transfer method into the whole heart through the coronary artery with hemagglutinating virus of Japan liposome. *J. Thoracic Cardiovasc. Surg.* 1997; **113**: 510–19.
48. Suzuki K, Sawa Y, Kaneda Y, Ichikawa H, Shirakura R, Matsuda H. *In vivo* gene transfection with heat shock protein 70 enhances myocardial tolerance to ischemia–reperfusion injury in rat. *J. Clin. Invest.* 1997; **99**: 1645–50.
49. Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.* 1992; **90**: 626–39.
50. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. *Circ. Res.* 1993; **73**: 1202–7.
51. Griscelli F, Opolon P, Chianale C *et al.* Expression from cardiomyocyte-specific promoter after adenovirus-mediated gene transfer *in vitro* and *in vivo*. *Comptes Rendus Acad. Sci.* 1997; **320**: 103–12.
52. Lesley A, Leinwand, Jeffrey Leiden M. Gene transfer approaches to myocardial diseases. In: Mockrin SC (ed.). *Molecular Genetics and Gene Therapy of Cardiovascular Diseases*. Marcel Dekker, New York, 1997; 487–528.
53. Muhlhauser J, Jones M, Yamada I *et al.* Safety and efficacy of *in vivo* gene transfer into the porcine heart with replication-deficient, recombinant adenovirus vectors. *Gene Ther.* 1996; **3**: 145–53.
54. Ellison KE, Bishopric NH, Webster KA *et al.* Fusigenic liposome-mediated DNA transfer into cardiac myocytes. *J. Mol. Cell. Cardiol.* 1996; **28**: 1385–99.
55. Silvestry SC, Taylor DA, Lilly RE *et al.* The *in vivo* quantification of myocardial performance in rabbits: A model for evaluation of cardiac gene therapy. *J. Mol. Cell. Cardiol.* 1996; **28**: 815–23.
56. French BA, Mazur W, Geske RS, Bolli R. Direct *in vivo* gene transfer into porcine myocardium using replication-deficient adenoviral vectors. *Circulation* 1994; **90**: 2414–24.
57. Barr E, Carroll J, Kalynych AM. Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. *Gene Ther.* 1994; **1**: 31–58.
58. Li JJ, Ueno H, Pan Y *et al.* Percutaneous transluminal gene transfer into canine myocardium *in vivo* by by replication-defective adenovirus. *Cardiovasc. Res.* 1995; **30**: 97–105.
59. Lamping KG, Rios CD, Chun JA, Ooboshi H, Davidson BL, Heistad DD. Intrapericardial administration of adenovirus for gene transfer. *Am. J. Physiol* 1997; **272**: H310–17.
60. Quinones MJ, Leor J, Kloner RA *et al.* Avoidance of immune response prolongs expression of genes delivered to the adult rat myocardium by replication-defective adenovirus. *Circulation* 1996; **94**: 1394–401.
61. Yang Y, Haecker SE, Su Q, Wilson JM. Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle. *Hum. Mol. Genet.* 1996; **5**: 1703–12.
62. Haecker SE, Stedman HH, BaliceGordon RJ *et al. In vivo* expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes. *Hum. Genet. Ther.* 1996; **7**: 1907–14.
63. Morsy MA, Caskey CT. Safe gene vectors made simpler. *Nature Biotechnol.* 1997; **15**: 17.
64. Fender P, Ruigrok RWH, Gout E, Buffet S, Chroboczek J. Adenovirus dodecahedron, a new vector for human gene transfer. *Nature Biotechnol.* 1997; **15**: 52–6.
65. Soonpaa MH, Daud AI, Koh GY *et al.* Potential approaches for myocardial regeneration. *Ann. N.Y. Acad. Sci.* 1995; **752**: 446–54.
66. Koh GY, Soonpaa MH, Klug MG. Long-term survival of AT_1 cardiomyocyte grafts in syngenic myocardium. *Am. J. Physiol.* 1997; **264**: H1727–33.
67. Koh GY, Klug MG, Soonpaa MH, Field LJ. Differentiation and long-term survival of C2C12 myoblast grafts in the heart. *J. Clin. Invest.* 1993; **92**: 1548–54.
68. Van Meter CH, Claycomb WC, Delcarpio JB *et al.* Myoblast transplantation in the porcine model: A potential technique for myocardial repair. *J. Thoracic Cardiovasc. Surg.* 1995; **110**: 1442–8.
69. Soonpaa MH, Koh GY, Klug MG, Field LJ. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science* 1994; **264**: 98–101.
70. Koh GY, Soonpaa MH, Pride HP, Cooper BJ, Zipes DP, Field LJ. Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dogs. *J. Clin. Invest.* 1995; **96**: 2034–42.
71. Doetschman T, Shull M, Kier A, Coffin JD. Embryonic stem cell model systems for vascular morphogenesis and cardiac disorders. *Hypertension* 1993; **22**: 618–29.
72. Qin L, Chavin KD, Ding Y *et al.* Retrovirus-mediated transfer of viral il-10 gene prolongs murine cardiac allograft survival. *J. Immunol.* 1996; **156**: 2316–23.
73. Barr E, Leiden JM. Systemic delivery of recombinant proteins by genetically modified myoblasts. *Science* 1991; **254**: 1507–9.
74. Campion DR. The muscle satellite cell: A review. *Int. Rev. Cytol.* 1984; **87**: 225–51.
75. Chiu RCJ, Zibaitis A, Kao RL. Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. *Ann. Thoracic Surg.* 1995; **60**: 12–18.
76. Yoon PD, Kao RL, Magovern GJ, Casscells W. Myocardial regeneration: Transplanting satellite cells into damaged myocardium. *Texas Heart Inst. J.* 1995; **22**: 119–25.
77. Schultz E, Albright DJ, Jaryszak DL, David TL. Survival of satellite cells in whole muscle transplants. *Anat. Rec.* 1988; **222**: 12–17.
78. Leor J, Patterson M, Quinones MJ, Kedes LH, Kloner RA. Transplantation of fetal myocardial tissue into the infarcted myocardium of rat: A potential method for repair of infarcted myocardium? *Circulation* 1996; **94**: II-332–6.
79. Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. *J. Clin. Invest.* 1996; **98**: 2512–23.
80. Kaneko Y, Ezure M, Tambara K, Inaba H, Furuse A. Cardiomyoplasty effectiveness: Review of the mechanism. *J. Cardiol.* 1996; **27**: 153–7.
81. Carpentier A, Chachques JC. Myocardial substitution with a stimulated skeletal muscle: First successful clinical case. *Lancet* 1985; **8498**: 97–8.
82. Tam SKC, Gu W, Nadal-Ginard B *et al.* Molecular cardiomyoplasty: Potential cardiac gene therapy for chronic heart failure. *J. Thoracic Cardiovasc. Surg.* 1995; **109**: 918–24.
83. Olson EN. Regulation of muscle transcription by the MyoD family. The heart of the matter. *Circ. Res.* 1993; **72**: 1–6.
84. Leor J, Prentice H, Sartorelli V, Quinones Patterson MJ, Kedes MLK, Kloner RA. Gene transfer and cell transplant: An experimental approach to repair a 'broken heart'. *Cardiovasc. Res.* 1997; **35**: 431–41.
85. Murry CE, Kay MA, Bartosek T, Hauschka SD, Schwartz SM. Muscle differentiation during repair of myocardial necrosis in rats via gene transfer with myod. *J. Clin. Invest.* 1996; **98**: 2209–17.

EXHIBIT C

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C12N 15/86, A61K 48/00 | A1 | (11) International Publication Number: WO 00/15821
(43) International Publication Date: 23 March 2000 (23.03.2000)

(21) International Application Number: PCT/US99/20730
(22) International Filing Date: 10 September 1999 (10.09.1999)
(30) Priority Data:
60/099,960 11 September 1998 (11.09.1998) US
(60) Parent Application or Grant
THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [/]; O. CHIEN, Kenneth, R. [/]; O. WANG, Yibin [/]; O. EVANS, Sylvia [/]; O. MUSICK, Eleanor, M. ; O.

Published

(54) Title: RECOMBINANT ADENOVIRUS FOR TISSUE SPECIFIC EXPRESSION IN HEART
(54) Titre: ADENOVIRUS RECOMBINANT POUVANT ACCOMPLIR UNE EXPRESSION SPECIFIQUE DU TISSU CARDIAQUE

(57) Abstract

The present invention relates to a human type-5 recombinant adenovirus vector for achieving cardiac restricted transcription involving utilization of the cardiomyocyte-restricted cardiac ankyrin repeat protein (CARP) promoter with inclusion of the inverted terminal repeat sequences from human adeno-associated virus (AAV). Using green fluorescent protein (GFP) as a marker gene, the recombinant adenovirus vector (Ad/CG/ITR) is shown to direct transgene expression to myocardial tissue in vivo and in vitro in mouse models.

(57) Abrégé

La présente invention concerne un vecteur d'adénovirus recombinant humain de type 5 pouvant accomplir une transcription cardiaque localisée, qui met en oeuvre le promoteur de la protéine de répétition de l'ankyrine cardiaque (CARP) localisée dans les cardiomyocytes, lequel promoteur est associé aux séquences de répétition terminale inversée issues du virus adéno-associé humain (AAV). L'utilisation de la protéine verte fluorescente (GFP) comme gène marqueur permet de voir que, chez les modèles murins, le vecteur d'adénovirus recombinant (Ad/CG/ITR) dirige l'expression transgénique vers le tissu du myocarde tant in vivo qu'in vitro.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/86, A61K 48/00 — A1

(11) International Publication Number: **WO 00/15821**

(43) International Publication Date: 23 March 2000 (23.03.00)

(21) International Application Number: PCT/US99/20730

(22) International Filing Date: 10 September 1999 (10.09.99)

(30) Priority Data:
60/099,960 11 September 1998 (11.09.98) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 9500 Gilman Drive Mail Code 0910, La Jolla, CA 92093–0910 (US).

(72) Inventors: CHIEN, Kenneth, R.; 6232 Calle Vera Cruz, La Jolla, CA 92037 (US). WANG, Yibin; 4142 Caminito Lita, San Diego, CA 92122 (US). EVANS, Sylvia; 2281 Via Apilia, Del Mar, CA 92014 (US).

(74) Agents: MUSICK, Eleanor, M. et al.; Brown, Martin, Haller & McClain, 1660 Union Street, San Diego, CA 92101–2926 (US).

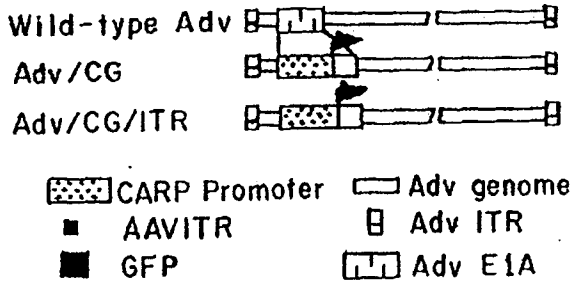
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: RECOMBINANT ADENOVIRUS FOR TISSUE SPECIFIC EXPRESSION IN HEART

(57) Abstract

The present invention relates to a human type–5 recombinant adenovirus vector for achieving cardiac restricted transcription involving utilization of the cardiomyocyte–restricted cardiac ankyrin repeat protein (CARP) promoter with inclusion of the inverted terminal repeat sequences from human adeno–associated virus (AAV). Using green fluorescent protein (GFP) as a marker gene, the recombinant adenovirus vector (Ad/CG/ITR) is shown to direct transgene expression to myocardial tissue *in vivo* and *in vitro* in mouse models.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Description

RECOMBINANT ADENOVIRUS FOR TISSUE SPECIFIC EXPRESSION IN HEART

This application claims the benefit of priority of United States Provisional Application Serial No. 60/099,960, filed September 11, 1998, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to a recombinant adenoviral vector construct and to methods for the study of gene function and gene therapy for heart disease and more specifically to methods of targeting tissue specific expression of a given transgene in cardiac tissue through use of inverted terminal repeat sequences from human adeno-associated virus.

BACKGROUND INFORMATION

Cardiovascular gene therapy represents a novel approach to the treatment of inherited and acquired heart disease. Gene transfer to the heart would allow for the replacement of defective or missing cellular proteins that are responsible for proper cardiac function. The control of *in vivo* cardiac function represents a complicated interplay between multiple genes, varied cell types, and environmental stimuli but the elucidation of this interplay remains dependent on a more complete understanding of the changes that occur at the molecular and cellular levels. Traditionally, the majority of human gene therapy protocols have relied on the *ex vivo* application of the therapeutic gene, through the introduction of a retroviral vector, to the affected cells or tissue. Because the *ex vivo* method of gene therapy depends on the removal from and reintroduction to the body of the target cells, the treatment of inaccessible or sensitive organs or tissue poses a major dilemma. The alternate strategy of direct *in vivo*

delivery of therapeutic genes to the target cells represents a preferable method of gene therapy.

Targeted gene expression in somatic tissues is essential for both gene therapy and *in vivo* analysis of gene function, mainly through the substitution of an affected gene, using a safe and effective delivery system for the therapeutic gene. To date, recombinant adenoviruses have replaced the retrovirus as an efficient gene delivery vector for a variety of cell types and tissues (Yeh, *et al.*, FASEB J 11, 615-23, 1997). Adenovirus vectors are highly efficient in the genetic modification of nondividing human cells and have the capacity to carry long segments of genetic information. The hurdle in using adenovirus as gene "delivery systems" is that when an adenovirus is administered to a patient to aid in the delivery of genes to specific cells, the patient's immune system may react against the virus. To overcome this hurdle, modifications have been made to make the adenoviral vector safer, less toxic to the cells and less likely to stimulate an immune response. This has involved removing the E1 region of the adenovirus gene which prevents the ability of the virus to express its own proteins required for making viral particles. In place of the E1 region, a therapeutic transgene can be inserted. The efficiency of this kind of exogenous gene delivery and subsequent expression can be high, as it does not normally integrate into the host genome, and it has a minimal effect on intrinsic host cell function (Baldwin, *et al.*, Gene Ther. 4, 1142-49, 1997). However, while adenoviral vectors are capable of producing high levels of transgene expression, their capacity to infect and program transgene expression in large numbers of cells and tissue, including the liver and lungs, poses limitations. As a result of this high level of transient infectivity, methods have been undertaken to direct transgene expression to specific tissues or areas of the body. For cardiac tissue, a number of attempts have been reported utilizing recombinant adenoviruses to achieve transgene expression in the heart through either intra-myocardial or intra-coronary injection (Brody, *et al.*, Ann. N.Y. Acad.

Sci. 716, 90-101, 1994; Barr, *et al.*, Gene Ther. 1, 51-8, 1994; Kypson, *et al.*, J. Thorac. Cardiovasc. Surg. 115, 623-30, 1998). While direct injection of viral particles into the myocardium or cardiac cavity have been shown to be more efficient for gene delivery to the myocardium, infection and transgene expression also occurs in non-cardiomyocytes, which causes speculation that any specificity of transgene expression that exists is achieved by targeted delivery rather than restricted transcription (Kass, *et al.*, Gene Ther. 1, 395-402, 1994; Kass, *et al.*, Methods Cell Bio. 52, 423-37, 1997). As a result, ectopic expression, particularly in liver and other tissue, remains a significant limitation for the generalized use of recombinant adenoviruses for gene transfer to specific cell types within the cardiovascular and other organ systems.

In most recombinant adenoviral vectors, the E1a region of the adenovirus genome, which encodes the protein with properties for transcriptional regulation, is deleted and replaced by a minigene "cassette" that typically includes a promoter of choice, the transgene coding region, and a polyadenylation signal (Yeh, *et al.*, FASEB J 11, 615-23, 1997). One possible approach to achieve tissue- specific transgene expression using adenoviruses is to employ cellular gene promoters that possess cell-type specificity at the transcriptional level, rather than commonly used viral gene promoters that have a high level of expression, but lack tissue specificity. In the past, a number of studies have utilized different cell promoters to achieve targeted transgene expression in various tissues, including smooth muscle (Kim, *et al.*, J. Clin. Invest. 100, 1006-14, 1997), pancreas (Dusetti, *et al.*, J. Biol. Chem. 272, 5800-4, 1997), endothelium (Morishita, *et al.*, J. Biol. Chem. 270, 27948-53, 1995), lung (Strayer, *et al.*, Am. J. Respir. Cell Mol. Bio. 18, 1-11, 1998), and several kinds of tumors (Su, *et al.*, Proc. Natl. Acad. Sci. USA 94, 13891-6, 1997; Siders, *et al.*, Cancer Res. 56, 5638-46, 1996). Similar attempts using cardiac-specific promoters such as the myosin light chain-2 (MLC-2v) and the alpha-myosin heavy chain (α-MHA) promoters, in the context

of adenoviruses, however, have not been wholly successful in providing tissue-restricted gene expression *in vivo* (Kim, *et al.*, J. Clin. Invest. 100, 1006-14, 1997). These results suggest that adenoviral genomic sequences surrounding the deleted E1a region may be responsible for at least partial specificity of the adjacent cellular promoter. It has also been suggested that sequences around the E1a region may contain negative regulatory elements that act in modulating the specificity and activity of a cellular promoter (Shi, *et al.*, Hum. Ther. 8, 403-10, 1997). This undesirable property of adenoviral vectors has limited their application, especially in the context of *in vivo* studies where tissue specific expression of the transgene is required.

Thus, the need remains for a transgene expression system utilizing recombinant adenoviral vectors that are tissue specific for use in *in vivo* and *in vitro* gene therapy and gene function analysis for both neonatal and adult subjects. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a human type-5 recombinant adenovirus vector to achieve cardiac restricted transcription in both neonatal and adult subjects utilizing the cardiomyocyte-restricted cardiac ankyrin repeat protein (CARP) promoter in cooperation with the inverted terminal repeat (ITR) sequences from human adeno-associated virus (AAV). Such a combination is effective in achieving cardiac tissue-specific transcription of the transgene both *in vitro* and *in vivo*.

The invention further provides a method to achieve tissue targeted expression of a given transgene in cardiac tissues in both neonatal and adult subjects. Such a method has significant applications in both gene function studies and gene therapy for inherited and acquired heart diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the constructs of recombinant adenovirus vectors. All recombinant adenovirus vectors were generated through homologous recombination between pJM17 plasmid DNA and the specific shuttle plasmid DNA in 293 cells.

Figure 2 shows a Northern-blot analysis of the relative cell-type specific transcription of GFP in cultured cells following adenovirus infection. RNA from uninfected, control and infected cardiac myocytes were subject to Northen-blot analysis using GFP coding sequences as a probe and normalized by hybridization signals for GAPDH mRNA.

Figure 3 shows a Southern-blot analysis of the relative cell-type specific transcription of GFP in cultured cells following adenovirus infection. DNA from control or infected cells were digested with NotI and XhoI restriction enzymes and the GFP expression was detected at approximately 3.0 kb size for Adv/CMV/GFP and 760 bases for Adv/CG/ITR.

Figure 4 shows a Northern-blot analysis of the level of GFP transcription in mouse heart and liver following intra-cardiac injection of adenovirus vectors.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a means for achieving cardiac restricted transcription of a transgene in both neonatal and mature cardiac tissues through the use of a recombinant adenoviral gene delivery vector which is engineered to contain a cardiomyocyte-restricted CARP promoter in conjunction with inverted terminal repeat sequences from human adeno-associated virus, the sequences of which are incorporated herein by reference. In the construction of adenovirus vectors, it is most common to delete the majority of the E1a and E1b regions of the serotype 5 adenovirus gene to prevent replication of the adenoviral DNA. A prototypical vector is constructed by inserting the desired exogenous

genetic information, including the left hand end inverted terminal repeat (ITR), signal enhancers, promoters for the expression of the desired exogenous gene, and a polyadenylation signal, into the former E1 position of the adenovirus. Fu, *et al.* (*Nat. Biotechnol.* 16, 253-7, 1998) incorporated herein by reference, have reported an unusual property of the inverted terminal repeat (ITR) sequences, specifically of adeno-associated virus (AAV). Adeno-associated viruses are satellite viruses derived from replication-deficient parvovirus and most often found in association with adenovirus or herpes simplex virus. The wild-type AAV is non pathogenic and can site specifically integrate into a host genome, can transduce nondividing cells, and does not induce an immune response which could destroy the transduced cells. Fu, *et al.* have shown that the inclusion of both the left and right end segments of the AAV-ITR sequences imparts the ability to enhance the level as well as tissue specificity of the transgene expression using viral gene promoters or tissue-specific cellular gene promoters in developing Xenopus embryos. Further, Philip, *et al.* (*Mol. Cell Bio.* 14, 2411-8, 1994) have demonstrated that the inclusion of both the left and right end AAV-ITR sequences in mammalian plasmid constructs results in the enhancement of efficiency and stability of transgene expression. In the context of a recombinant adenovirus vector, inclusion of both the left and right end ITR sequences from adeno-associated virus has the ability to enhance tissue specificity of the exogenous transgene expression when a cardiac restricted promoter is utilized.

In order to achieve targeted gene expression in the cardiac tissue, the 213 base pair, 5' flanking promoter fragment of the CARP gene was selected to direct the transgene expression. Three separate lines of transgenic mice were created which harbored various CARP promoter/ β-galactosidase reporter genes for the purpose of studying this 5' flanking CARP promoter. CARP, a cardiac ankyrin repeat protein, is a putative downstream regulatory gene in the homeobox gene Nkx2-5 pathway

which regulates the expression of the ventricular myosin light chain-2 (MLC-2v) gene (Zou, *et al.*, *Development* 124, 793-804, 1997). Studies have identified an essential GATA-4 binding site in the proximal upstream regulatory region of the CARP gene and cooperative transcriptional regulation mediated by Nkx2.5 and GATA-4. This cooperative regulation is dependent on the binding of GATA-4 to its cognate DNA sequence in the promoter, which suggests that Nkx2.5 may exert its control on the CARP promoter, at least in part through GATA-4. As used herein, the term "homeobox gene Nkx2-5" refers to the murine homologue of Drosophila gene *tinman* which has been previously shown to be required for heart tube looping morphogenesis and ventricular chamber-specific myosin light chain-2 expression during mammalian heart development. Ventricular myosin light chain-2 (MLC-2v), one of the earliest markers of ventricular regionalization during mammalian cardiogenesis, has been the subject of numerous studies seeking to identify the molecular pathways that guide cardiac ventricular specification, maturation and morphogenesis. These studies have identified a 28 base pair HF-1a/MEF-2 *cis*-element in the MLC-2v promoter region which appears to confer the cardiac ventricular chamber-specific gene expression during cardiogenesis as well as showing that the ubiquitous transcription factor YB-1 binds to the HF-1a site in conjunction with a co-factor. Moreover, data further indicates that regulatory elements within the 5' flanking region of the CARP gene are capable of directing region-specific (atrial vs. ventricular and left vs. right) transgene expression in the heart. The 213 base pair sequence element in the 5' flanking region of the CARP gene appears to be sufficient to confer conotruncal-specific transgene expression.

CARP forms a physical complex with YB-1 in cardiac myocytes and endogenous CARP seems to be localized in the cardiac myocyte nucleus. Zou, *et al.* (*Development* 124, 793-804, 1997) have demonstrated that CARP can negatively regulate HF-1-TK minimal promoter activity in an HF-1 sequence-dependant manner in cardiac myocytes as well as displaying

transcriptional inhibitory activity when fused to a GAL4 DNA-binding domain in both cardiac and non-cardiac cells. Analysis using a standard Northern-blot protocol indicates an enriched level of CARP mRNA in the myocytes of cardiac tissue, and to a lesser degree in skeletal muscle, and that endogenous CARP expression can be upregulated in heart and other tissue upon induction of cytokine activity (Chu, *et al.*, *J. Biol. Chem.* 270, 10236-45, 1995; Jeyaseelan, *et al.*, *J. Biol. Chem.* 272, 22800-8, 1997).

Cytokines play a critical role in the control and maintenance of the signaling pathways that regulate mammalian physiology in multiple organ systems. Their widespread importance is reflected in the extensive tissue distribution of cytokine networks, where a deficiency in cytokine signaling components can result in multiple organ defects. In a study by Hirota, *et al.* (*Cell 97*, 189-198, April 16, 1999) incorporated herein by reference, researchers explored the role of IL-6 related cytokines in the pathogenesis of cardiac failure, which is the leading cause of combined morbidity and mortality in the United States and other developed countries. In response to chronic increases in blood pressure and blood volume overload, as is common in myocardial injury, the heart responds by becoming enlarged in order to maintain normal cardiac function, a process known as compensatory hypertrophy. CT-1, a member of the IL-6 cytokine family, can activate the onset of myocyte hypertrophy in vitro and has been shown to be vital as a potent myocyte survival factor in cardiac muscle cells by blocking the onset of cardiomyocyte apoptosis. There is further evidence that the presence of cytokine receptor gp130 expression in cardiac myocytes can lead to compensatory cardiac hypertrophy, thus delaying the onset of cell apoptosis and ultimately, heart failure. A deficiency in the gp130 cytokine receptor signaling pathway often results in severe cardiac defects in developing embryos possibly leading to an early lethality *in utero*. A therapeutic strategy of introducing the transgene coding region of gp130 directly into the embryonic heart cells

using the tissue specific adenoviral vector delivery system of the present invention, while still *in utero*, may be a viable treatment option. Similarly, introduction of the gp130 gene into mature cardiac myocytes under constant biomechanical stress, through attachment to the cardiac specific CARP promoter of the present invention, may initiate expression of the gp130 cytokine receptor pathway, resulting in enhanced cardiac compensatory hypertrophy, offsetting cardiomyocyte apoptosis, and thus averting cardiac failure.

Generation of recombinant adenovirus vectors

The recombinant adenovirus vector of the present invention was constructed through homologous recombination between shuttle plasmid DNA containing the transgene and pJM17 plasmid DNA containing the entire genome of the human type-5 adenovirus, the method of construction described by Wang, et al., *J. Biol. Chem.* 273, 2161-8, 1998, for the generation of Adenovirus/CMV vectors. The *E. coli* host containing plasmid pJM17 that includes DNA of the entire genome replication defective human type-5 adenovirus has been deposited as ATCC Accession No._____ in the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A., under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted.

The shuttle plasmid, pAdv/CARP, was assembled with a 2.5 kilobase CARP promoter, excised from the 5' flanking region of the CARP gene and inserted between the Bam H I and Xho I sites of pXCJL.2. (The *E. coli* host containing plasmid pJM17 including DNA of the entire genome of the human type-5 adenovirus containing the insert murine CARP promoter sequence has been deposited as ATCC Accession No._____.) The resulting construct was shown to be sufficient to confer cardiac-restricted marker gene expression in cultured cells and transgenic mice. (See Zou, *et al.* (*Development* 124, 793-804, 1997).)

With the elucidation of CARP function, this 2.5 kilobase CARP promoter was thus used to generate an adenovirus/CARP/marker construct, using a green fluorescent protein (GFP) gene as a visual reporter for identification of adenovirus/CARP promoter activity following *in vitro* and *in vivo* administration of the adenovirus construct. To construct the reporter gene, GFP coding sequences were excised from pEGFP-N1 (Clontech, CA) through Bam HI and Afl III digestion, and inserted into the Xho I site of pAdv/CARP to generate pAdv/CG. The resulting recombinant adenovirus was designated Adv/CG.

In order to determine whether inclusion of AAV ITR sequences in the adenovirus genome has the ability to enhance tissue specific expression of the transgene, the DNA fragment containing the CARP promoter and GFP coding sequences was removed from pAdv/CG through Bam HI and Sal I digestion and subsequently inserted into the Xho I site of the pAdv/AAV plasmid, which is derived from pXCJL.2 containing two copies of the AAV ITR sequence. The resulting plasmid, pAdv/CG/ITR, was used to generate a recombinant adenovirus, designated as Adv/CG/ITR, using transformation techniques known to those in the art. Figure 1 provides a diagrammatic representation of the recombinant adenovirus constructs. All recombinant adenovirus vectors were plaque-purified using standard methods and analyzed by PCR for the presence of the transgene in the viral genome. High titer viral stocks were prepared

by a single ultracentrifugation on a CsCl gradient as described by Wang, et al., *J. Biol. Chem.* 273, 2161-8, 1998, the technique well known in the art.

Cardiomyocytes and cardiac fibroblast culture and adenovirus infection – To establish the cardiac tissue specificity of the adenoviral vector of the present invention, primary ventricular myocytes and cardiac fibroblasts were prepared from 1 - 2 day old Sprague-Dawley rats using a Percoll gradient method as described by Iwaki, et al., *J. Biol. Chem.* 265, 13809-17, 1990. Cardiac fibroblasts were isolated from the upper band of the Percoll gradient, and subsequently plated in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Myocytes were isolated from the lower band of the Percoll gradient and subsequently plated in 4:1 Dulbecco's modified Eagle's medium; 199 medium, 10% horse serum and 5% fetal bovine serum. The cardiac fibroblasts and myocytes were infected with the recombinant adenovirus at varied multiplicity of infection (M.O.I.) 24 hours after isolation and were then incubated for an additional 48 hours before being subject to DNA, RNA, and fluorescent photomicroscopic analysis.

RNA and DNA analysis

RNA samples were prepared from cultured cells and mouse tissues using RNAzol B solution according to the manufacturer's protocol (TEL-TEST, Texas). Northern blot hybridization was performed according to a standard protocol, familiar to those of skill in the art, using GFP coding sequences to generate a P^{32} labeled probe. Total DNA, purified from cultured cells and mouse tissues, were prepared using the protocol as directed by a Purogene DNA isolation kit, and then digested with the restriction enzymes Xho I/Not I for Southern blot analysis using the same P^{32} labeled GFP coding sequence probes as used in the Northern blot hybridization.

In vivo adenoviral injection into neonatal mouse heart

Using the procedure of high efficiency, long term expression via adenoviral vector injection into neonatal mouse as described by Brody, et al., *Ann. N.Y. Acad. Sci.* 716, 90-101, 1994, 1-day old mouse neonates were anesthetized by hypothermia at 4 °C for 2 minutes. 10 μl of viral solution, containing 2 x 10^9 viral particles, were injected directly into the cardiac cavity using a flame stretched capillary tube mounted on a micromanipulator. Flashback of pulsatile blood in the capillary tube gave positive indication of correct intracavitary placement. The subject neonatal mice were allowed to recover by rewarming at room temperature and were then placed back with the mother for a 48 hour period. At the end of the 48 hours, the neonatal mice were sacrificed, and the heart and liver were removed from the body for DNA, RNA and fluorescent photomicrographic analysis.

Mouse embryo culture and microinjection of adenovirus vector

The preparation of rat serum was by the method as described by Cockroft, et al., *Dissection and Culture of Post-Implantation Embryos*, 1990 (IRL Press, Oxford, England). Whole mouse embryos were cultured according to the method of Sturm and Tam, Methods Enzymol. 225, 164-90, 1993. As per the protocol, timed pregnant female mice were sacrificed by cervical dislocation. The uterus was dissected from the body and rinsed in phosphate buffered-saline (PBS) to remove any residual blood and then transferred to a sterile receptacle containing PB1 media (137 mM NaCl; 2.7 mM KCl; 0.5 mM $MgCl_2$; 8.04 mM Na_2HPO_4, 1.47 mM KH_2PO_4; 0.9 mM $CaCl_2$; 0.33 Na pyruvate; 1g/L glucose; 0.01g phenol red, pH 7.35; 100 ml/L streptomycin; 100 U/ml penicillin; all reagents from Sigma Biochemicals, St. Louis, MO.). Embryos of 11 days post coitum (E11) were dissected from the uterus and the decidual and Riechert's membrane removed. The embryos were separated from the yolk sac and amnion, which had been left attached during dissection to

ensure continuity of the vessels connecting the embryo to the yolk sac or the umbilical vessels from the embryo to the placenta. The isolated embryos were then transferred to pre-equilibrated media (consisting of 50% rat serum which was continuously gassed (95% O_2, 5% CO_2)) in roller culture bottles placed on a rocker table and incubated at 37°C. After one hour in culture, the embryos were placed in a petri dish and microinjected into the left ventricle using a 6 μm diameter glass pipette. The micropipettes had been previously prepared using a multistage pipette puller (Suter Instrument Co., Novato, CA) to pull 1 mm glass capillary tubes into the 6 μm needle configuration. Each micropipette was attached to a MX-110-R 4 axis, manual micromanipulator (Newport Instruments, Newport, CA) using electrode holders. Intracardiac injection of 1 μl of a high titer viral solution (2 x 10^8) proceeded at a low-flow rate, on the order of 0.2 to 0.5 μl per second (2 to 5 seconds for one microliter.)

The ability to target transgene expression in *in vivo* cardiomyocytes represents a new and powerful approach to study and manipulate specific gene function during the process of cardiac development as well as the treatment of heart disease using gene therapeutic technology. The strategy of using a cardiac-restricted cellular promoter in combination with both the right and left hand ITR sequences from AAV (SEQ ID NO:1 and SEQ ID NO:2, respectively) to achieve cardiac specific transgene expression in both embryonic and post-natal heart tissue distinguishes the present invention from other recombinant adenoviral vectors currently found in the art. Further, the inclusion of both AAV-ITR sequences in the context of a cardiac-restricted recombinant adenovirus vector preserves the tissue-specificity of the cellular promoter activity both *in vitro* and *in vivo* and, when combined with a targeted delivery system, makes the present invention significant as gene based therapy to treat heart disease

as well as providing a method to study specific gene function in embryonic and post-natal heart.

As previously reported in the studies of Fu, *et al.* and Phillip, *et al.*, the presence of AAV-ITR sequences in mammalian cell systems, as well as in developing Xenopus embryos, has the effect of enhancing transgene expression. The reports of studies of Fu, *et al.* and Phillip, *et al.* are incorporated herein by reference. While experiments in Xenopus embryos suggest that ITR sequences facilitate DNA segregation among replicating cells, other studies implicate AAV- ITR sequences in enhancing genomic integration after transfection, at least in an *in vitro* setting.

Regardless of the mode of action, adenovirus DNA remains mostly in episomal form in infected cells. Since cardiac myocytes, on their own, do not demonstrate robust replication after birth, it is unlikely that these two properties contribute significantly to the enhancement of tissue specificity in heart tissue. An alternative mechanism that has also been implicated in Xenopus studies is that AAV-ITR has insulating properties that shield the flanked transgene from the effects of other regulatory elements within the adenoviral genome. In fact, this mode of action has support from findings establishing the existence of negative regulatory elements located around the adenovirus E1a region that can modulate the specificity of the adjacent cellular promoter. Two previous studies from Franz, *et al.* (Cardiovasc. Res. 35, 560-6, 197) and Rothman, *et al.* (Gene Ther. 3, 919-26, 1996) have also reported the generation of cardiomyocyte-specific adenoviruses using the MLC-2v promoter but not with α-MHC promoter even though both promoters have cardiomyocyte-specific transcriptional activity. The reports of studies of Franz, *et al.* and Rothman, *et al.* are incorporated herein by reference. The lack of transgene expression of Adv/CG (CARP promoter without AAV ITR) indicates that the specific transcriptional activity of a cellular promoter is subject to significant influence by the surrounding adenovirus genome.

Therefore, inclusion of AAV ITR provides a general strategy to achieve tissue-specific transcription using other cellular promoters.

Hammond, *et al.* (U.S. Patent No. 5,792,453) have reported a replication defective adenovirus vector comprising a transgene coding for an angiogenic protein or peptide that can be targeted to the myocardium of a patient by intracoronary injection directly into the coronary arteries, for the treatment of myocardial ischemia. In order to deliver these angiogenic proteins, which may include aFGF, bFGF, FGF-5 (fibroblast growth factors) and VEGF (vascular endothelial growth factor), Hammond, *et al.* rely on ventricular myocyte-specific promoters, namely the promoters from MLC-2v and α-MHC, to achieve targeted delivery. However, as has been established by the method of the present invention, myocardial expression of the angiogenic transgene in the cardiomyocytes is more likely the result of direct cardiac application of the adenoviral vector rather than the use of the MLC-2v or α-MHC promoters. In addition to the CARP gene promoter (SEQ ID NO: 3), the AAV-ITR sequences (SEQ ID NOS: 1 and 2) of the present invention can be used with other cardiac restricted promoters, including:

1. α-myosin heavy chain gene
2. ß-myosin heavy chain gene
3. Myosin light chain 2v gene
4. Myosin light chain 2a gene
5. CARP gene
6. Cardiac α-actin gene
7. Cardiac m2 muscarinic acetylcholine gene
8. ANF
9. Cardiac troponin C
10. Cardiac troponin I
11. Cardiac troponin T
12. Cardiac sarcoplasmic reticulum Ca-ATPase gene
13. Skeletal α-actin
14. Artificial cardiac promoter derived from MLC-2v gene

The AAV-ITR sequences can also be used to generate other target vectors for conditional gene expression by using inducible promoters. The inclusion of the AAV-ITR sequences of the present invention, in the

adenoviral vector of Hammond, *et al.* would assure the tissue specific expression of the angiogenic transgene and, thus, avoid the negative effects these angiogenic proteins have on other tissues in the body.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE 1

Cell-type specific transcription mediated by Adv/CG/ITR vector in cultured cells

This example provides an evaluation of transcriptional specificity of the recombinant adenovirus containing the cardiomyocyte enriched CARP promoter coupled (SEQ ID NO: 3) with the inverted terminal repeat sequences (ITR) from human adeno-associated virus (AAV) (SEQ ID NOS: 1 and 2).

Purified adenoviral vectors were used to infect cultured primary cardiac fibroblasts and ventricular myocytes prepared from neonatal rat heart. An adenovirus vector with a human cytomegalovirus (CMV) enhancer/promoter driving GFP expression (Adv/CMV/GFP) was used as a positive control for viral infection and GFP detection. As previously reported by Wang, et al., *J. Biol. Chem.* 273, 2161-8, 1998, recombinant adenoviruses are capable of efficiently infecting many cell types, including cardiomyocytes, at a low multiplicity of infection (M.O.I.) of less than 100 viral particles/cell and the expression of GFP can be readily detected at a high level in more than 95% of cardiomyocytes cultured from neonatal rat hearts. Cardiac fibroblasts, however, require an M.O.I. of more than 1,000 viral particles/cell in order to achieve approximately 70% of infection. Using the same level of viral infection (100 or 1,000 viral particles/cell), GFP expression was not detected in either myocytes or fibroblasts infected with the Adv/CG vector. In contrast, when the Adv/CG/ITR vector was used as the infecting agent, GFP expression was observed in more than 90% of the cardiac myocytes,

but not at any appreciable levels in cardiac fibroblasts. These results demonstrate that the cardiac specific CARP promoter/AAV-ITR is necessary to achieve transcriptional specificity of the transgene in the ventricular myocytes of cultured neonatal rat heart while transcriptional expression is not found in the fibroblasts even at even high M.O.I.

Further evaluation of cardiac-restricted expression of GFP by Adv/CG/ITR at the transcriptional level was performed using a standard Northern-blot protocol for mRNA detection. As seen in Figure 2, the levels of GFP mRNA in Adv/CMV/GFP infected cardiomyocytes and cardiac fibroblasts are readily detectable. In Adv/CG infected cells, however, the GFP mRNA was not detected, which was in agreement with the observations from evaluation by fluorescent photomicroscopy. In contrast, RNA samples from cardiomyocytes infected with Adv/CG/ITR showed significant levels of GFP transcript, while RNA samples from infected cardiac fibroblasts has significantly lower levels of GFP.

To ensure that the observed cardiomyocyte restricted expression of Adv/CG/ITR vector was at the transcriptional level rather than secondary to an effect of infectivity, a standard Southern-blot analysis was performed using DNA samples from infected fibroblasts and myocytes. As seen in Figure 3, viral DNA was present at comparable levels in both cardiomyocytes and fibroblasts infected with either Adv/CMV/GFP or Adv/CG/ITR vectors. These results confirm that the transcriptional activity of the CARP promoter is suppressed in the context of the adenoviral genome and that the inclusion of ITR sequences from AAV allows retention of cardiac restricted cell-type specificity of the CARP promoter in cultured cells.

EXAMPLE 2

In vivo cardiac restricted transgene expression mediated by the Adv/CG/ITR vector in neonatal mouse heart

In order for the present invention to be viable as a method of gene therapy for the treatment of inherited and acquired heart disease, it is

important to establish that cell type specificity of the Adv/CG/ITR vector, demonstrated *in vitro*, can also direct tissue targeted transgene expression *in vivo*. To test this, approximately 2 x 10^9 adenovirus particles were injected directly into the heart muscle of day-old mice. Following direct administration of Adv/CMV/ITR vectors into the cardiac cavity, the level of infection was measured to be approximately 10% with a distribution concentrated primarily in the epicardium of the ventricular wall. In addition, a high level of GFP expression was also detected in the liver of the infected animals. This observation agrees with many earlier published studies where it has been established that the delivery of the recombinant adenovirus through the systemic circulation always lead to high levels of infection in the liver and other non-cardiac tissue. Similar to previous observations, direct intracardiac injection of the Adv/CG vector resulted in no detectable GFP in any tissue, including the heart. As predicted, the adenoviral vector of the present invention, Adv/CG/ITR, gave rise to a significant level of GFP expression in heart tissue but a much lower expression in liver and other non-cardiac tissue.

To further evaluate tissue specific expression of the transgene, Northern-blot analyses were performed on RNA samples prepared from the heart and liver of the infected mice. The results of the analysis is shown in Figure 4. In Adv/CMV/GFP injected animals, GFP mRNA was detected at high levels in both the heart and liver confirming the results generated by the Northern-blot analysis. In the Adv/CG/ITR injected mice, however, GFP mRNA was detected primarily in the heart and at a significantly lower level in the liver. The inclusion of AAV ITR in the adenovirus vector, as prescribed in the present invention, enhances the tissue-specificity of transgene expression in vivo, making the adenovirus vector of this invention suitable for use in the delivery of gene therapeutic agents.

EXAMPLE 3

Cardiac-restricted transgene expression mediated by the Adv/CG/ITR vector in cultured mouse embryos

The tissue-specific gene transfer properties of the present invention can also be applied to study gene function during embryonic cardiac development. To demonstrate the ability of targeted gene expression, in developing heart tissue, using tissue specific adenoviral vectors, approximately 2 x 10^8 particles of each of the recombinant adenovirus vectors, Adv/CMV/GFP, Adv/CG and Adv/CG/ITR were microinjected into the cardiac cavities of developing mouse embryos at 11 days post coitum. Following an additional 25 hours of culturing after initial injection of the adenoviral vectors, GFP expression was evaluated. Injection of the Adv/CMV/GFP vector resulted in high relative levels of GFP expression in the developing heart as well as in a wide range of other tissues. This wide spread expression pattern confirms earlier evidence indicating that the Adv/CMV/GFP vector is capable of directing transgene expression in a broad range of tissues and that transgene expression is most likely dictated by the distribution of viral particles in the developing embryo. Following injection of the recombinant Adv/CG vector, analysis by fluorescent photomicroscopy revealed no GFP expression in any part of the embryo which correlated with *in vitro* results derived from cultured cells and *in vivo* data from neonatal mice studies. Injection of Adv/CG/ITR vector gave rise to the expression of GFP in cardiac tissue with no ectopic expression, detectable by fluorescent photomicroscopy, in other tissues. Specifically, GFP expression was at the highest level in the atrium .

These results demonstrate that inclusion of the ITR sequences from AAV, as in the Adv/CG/ITR vector construct of the present invention, eliminates ectopic expression of the transgene, and allows for cardiac tissue specific expression, following direct ventricular injection of the adenoviral vector into developing embryos. Such tissue specific expression, directed by the Adv/CG/ITR vector of the present invention, can be applied to the development of other recombinant adenoviral vectors that contain ITR sequences from AAV and may confer cardiac

specific expression of a therapeutic transgene in the treatment of cardiac damage and dysfunction.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims:

Claims

What is claimed is:

1. A human type-5 recombinant adenovirus vector which has tissue specific transcription of a transgene, the adenovirus vector comprising;

a tissue-restricted promoter; and

inverted terminal repeat sequences from human adeno-associated virus (AAV).

2. The human type-5 recombinant adenovirus vector of claim 1, wherein the tissue-restricted promoter is a cardiac-restricted promoter.

3. The human type-5 recombinant adenovirus vector of claim 1, wherein the tissue specificity is for cardiac tissue.

4. The human type-5 recombinant adenovirus vector of claim 1, wherein the inverted terminal repeat sequences from AAV comprise two copies of the inverted terminal repeat sequence.

5. The human type-5 recombinant adenovirus vector of claim 4, wherein the two copies of inverted terminal repeat sequence from AAV comprise the left end and right end inverted terminal repeat sequence.

6. The human type-5 recombinant adenovirus vector of claim 5, wherein the left end and right end inverted terminal repeat sequence from AAV comprise the 5' end and the 3' end inverted terminal repeats respectively.

7. The human type-5 recombinant adenovirus vector of claim2, wherein the cardiac-restricted promoter comprises a cardiac-restricted promoter from the group consisting of α-myosin heavy chain gene, β-myosin heavy chain gene, myosin light chain 2v gene, myosin light chain 2a gene, CARP gene, cardiac α-actin gene, cardiac m2 muscarinic acetylcholine gene, ANF, cardiac troponin C, cardiac troponin I, cardiac troponin T, cardiac sarcoplasmic reticulum Ca-ATPase gene, skeletal α-actin, and artificial cardiac promoter derived from MLC-2v gene.

8. The human type-5 recombinant adenovirus vector of claim 7, wherein the cardiac restricted promoter is a cardiomyocyte-restricted ankyrin repeat protein (CARP) promoter.

9. A method for targeted gene therapy for heart disease comprising combining a cardiac-restricted cellular promoter with inverted terminal repeat sequences from adeno-associated virus.

10. The method for targeted gene therapy for heart disease of claim 9, wherein the inverted terminal repeat sequences from AAV comprise two copies of the inverted terminal repeat sequence.

11. The method for targeted gene therapy for heart disease of claim 9, wherein the two copies of inverted terminal repeat sequence from AAV comprise the left end and right end inverted terminal repeat sequence.

12. The method for targeted gene therapy for heart disease of claim 9, wherein the left end and right end inverted terminal repeat sequence from AAV comprise the 5' end and the 3' end inverted terminal repeats respectively.

13. The method for targeted gene therapy as in claim 9, wherein the cardiac-restricted promoter comprises a cardiac-restricted promoter from the group consisting of α-myosin heavy chain gene, β-myosin heavy chain gene, myosin light chain 2v gene, myosin light chain 2a gene, CARP gene, cardiac α-actin gene, cardiac m2 muscarinic acetylcholine gene, ANF, cardiac troponin C, cardiac troponin I, cardiac troponin T, cardiac sarcoplasmic reticulum Ca-ATPase gene, skeletal α-actin, and artificial cardiac promoter derived from MLC-2v gene.

14. A method for the evaluation of gene function comprising combining a cardiac-restricted cellular promoter with inverted terminal repeat sequences from adeno-associated virus.

15. The method for the evaluation of gene function of claim 14, wherein the cardiac-restricted cellular promoter is a CARP promoter.

16. The method for the evaluation of gene function of claim 14, wherein the cardiac-restricted cellular promoter is a CARP promoter containing a marker gene.

17. The method for the evaluation of gene function of claim 16, wherein the marker gene comprises a green fluorescent protein gene.

18. The method for the evaluation of gene function of claim 14, wherein the inverted terminal repeat sequences from AAV comprise two copies of the inverted terminal repeat sequence.

19. The method for the evaluation of gene function of claim 14, wherein the two copies of inverted terminal repeat sequence from AAV comprise the left end and right end inverted terminal repeat sequence.

20. The method for the evaluation of gene function of claim 9, wherein the left end and right end inverted terminal repeat sequence from AAV comprise the 5' end and the 3' end inverted terminal repeats respectively.

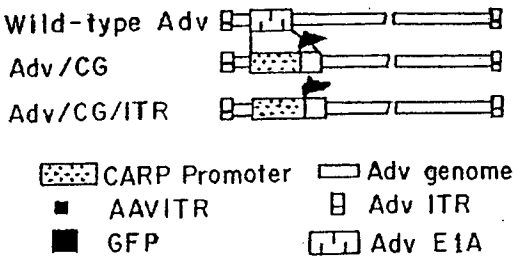


FIG. 1

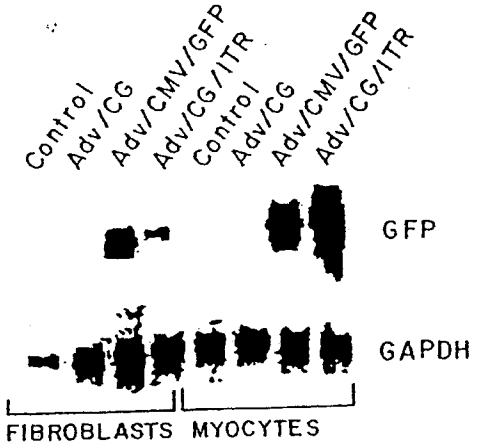


FIG. 2

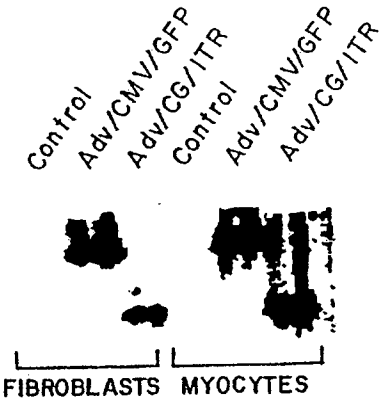


FIG. 3

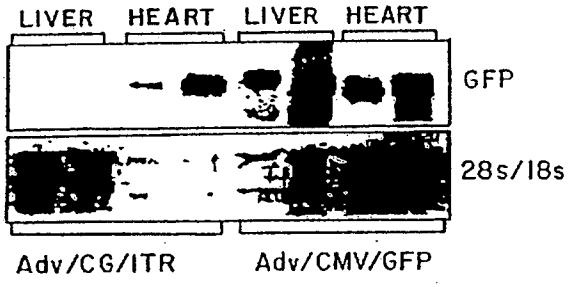


FIG. 4

SEQUENCE LISTING

<110> Kenneth Chien
Yibin Wang
Sylvia Evans
<120> NOVEL RECOMBINANT ADENOVIRUS FOR TISSUE SPECIFIC EXPRESSION IN HEART
<130> 6627-8045
<140> unknown
<141> September 10, 1999
<150> US 60/099,960
<151> September 11, 1998
<160> 3
<170> Word Perfect 8.1

<210> 1
<211> 174
<212> ssDNA
<213> adeno-associated virus 2; Viruses; ssDNA viruses; Parvoviridae; Parvovirinae; Dependovirus

<220>
<221> enhancer; 5' inverted terminal repeat
<222> 1...174

<400> 1

ggccactccc	tctctgcgcg	ctcgctcgct	cactgaggcc	gcccgggcaa	50
agcccgggcg	tcgggcgacc	tttggtcgcc	cggcctcagt	gagcgagcga	100
gcgcgcagag	agggagtggc	caactccatc	actaggggtt	cctggagggg	150
tggagtcgtg	acgtgaatta	cgta			174

<210> 2
<211> 183
<212> ssDNA
<213> adeno-associated virus 2; Viruses; ssDNA viruses; Parvoviridae; Parvovirinae; Dependovirus

<220>
<221> enhancer; 3' inverted terminal repeat
<222> 1...183

<400> 2
catggctacg tagataagta gcatggcggg ttaatcatta actacaagga 50
acccctagtg atggagttgg ccactccctc tctgcgcgct cgctcgctca 100
ctgaggccgg gcgaccaaag gtcgcccgac gcccgggctt gcccgggcg 150
gcctcagtga gcgagcgagc gcgcagagag gga 183

<210> 3
<211> 2247
<212> mRNA
<213> Mus musculus; Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.

<220>
<221> promoter
<222> 1-2247

<300>
<301> Zou, Y., et al.
<302> CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway
<303> Development
<304> 124
<305> 4
<306> 793-804
<307> 1997

<400> 3
nagctcncat gcctgcaggt cgactctaga ggatcctttc atgtttaaca 50
atatcaaccc taacccaagg ggaacagcct gcctgacagt ggctttgcca 100
cccatgaata cttcctagtc tagtccgttt gtgaaactca gcccatccca 150
acacttctgc aagccccatc ctctacaagg tgctcattgg gaatttcctg 200
gagcttctct ttcaggatca gcctgattct agggcagcag ttctcaacct 250
gggggcctcg acccctttgg gggaatcaaa cgacccttta caggggtcac 300
atatcatcta tcctatatgt caggtattta cattacgatt cgtaacagta 350
gcaaaattac aggtatgaaa tagcaatgaa ataatttttat gattgaaggt 400
caccacaaca tgaggccgcc acactgttct agagaaaaat cacctgggtg 450
gggaaaggtt tgggaaagcc tttctgtcca ttcttcattc ttcaaagtga 500
tgtgttcaca gaaagccttt cagctgttct gctggggctc ttagtaagtc 550
tgagtaggaa ctgtatgtac caggtctgct tcttatgggt ggagccaaga 600
cgcatcgtgg gtggagcgaa gacgcaacct caccttctac tctgcatcca 650
tagcaagtag cctaatgttc tgngtctagg gtcatctctg tgaatcgaga 700
tccttggccc ttgtttgaat tagggaggca caaaatctta aaaaattcaa 750
gactgntcaa caanccanaa gtcctttctc aaaaggaaag gncttaactn 800

tnancccccc	tttacttttg	agtcaaggcc	tggaaccaaa	ccggccccag	850
gaatgaaaaa	agcttgccat	nacctggttg	gccccttna	snaggncaaa	900
aaaaaattgt	ggttaacntt	gaaaaaccga	agaccaacag	ttatcctcta	950
gaaacacaat	ttgctggttg	aacagctgaa	gtggggtggg	ggttcttacc	1000
ccatgttcat	ggaagggtga	gtgaggagag	acagatatat	gaggccagca	1050
taacaaacat	acacaacacc	ctaattaaca	cttccctctt	ctactgacac	1100
ccccttcact	ctcctctttc	ataaaaaata	aaaaaagtat	tttagtggct	1150
cttacgatag	aatctttcct	cgaactataa	aaagatctaa	atatttatat	1200
ttttcacatt	ttaatatctt	agcgatgaca	agccagaaac	aagatttttt	1250
gcctctctca	acagcaaagc	ttggggcctt	tttgtttccg	tgttaggaat	1300
agaacacgag	agccccgtgt	atctaggcag	atgctctatc	attagcccat	1350
gagtctccag	cctcagacgc	acatttttct	cgggctctct	taagcttttc	1400
ccacagcatt	gggaaacttt	actgacagca	tccaagttgt	gcttctgcta	1450
agaactggac	tcacatctct	ctggcatcac	ttcggcccgt	tttggggtag	1500
atcctctgat	tagccttcag	atttagaaca	cggtgagcct	gtggtcacta	1550
attatggcca	gtgacaccat	agagtcaaag	tgcattactg	aatgctttca	1600
atttctccta	atgctggtac	gatggcatgt	cacagggcca	ttttagctgc	1650
agacatcatc	cagagaattc	caaacagata	ggacaagtgg	cacccagacc	1700
catctccttc	ccctcgggct	gattatcccc	aaaataggat	gtcccaaagc	1750
aacacttccc	agccaactgg	agtgctgata	agtccagtta	tcagaaagat	1800
atggctgtaa	gtgtgatgca	cagtgcttgc	attttcttga	tacgttagtc	1850
atatgagagc	tgacaaagaa	ggaaaaagag	cagcgatgtg	tgcaatatta	1900
acaggcagct	gtcccctggc	ttcccgatac	gtgggatgac	tcgcattgct	1950
gagcggtgtg	gtcactgcca	aaggaatgac	cctctcacat	ttcttcctga	2000
ttcgcatacg	ccgcggccag	cttgtcatct	ccctcttggg	cttcccagac	2050
actaagtctg	gaatgaaaat	tcacctgcct	ctgaattggc	cactggtggg	2100
agcaggggtg	tgacttggct	tcccaggctg	gaagattatc	tcacccagcc	2150
ctactatata	acgggctggt	gtggaggggc	tccacagggc	cagttccagg	2200
ggttcatcca	caagagagaa	aaacatagac	tcacggctgc	caacatg	2247

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/20730

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 13598 A (UNIV PENNSYLVANIA) 9 May 1996 (1996-05-09) page 9, line 1 -page 29, line 21; examples 1,8	1-7, 9-14, 17-20
Y	ROTHMANN T ET AL: "HEART MUSCLE-SPECIFIC GENE EXPRESSION USING REPLICATION DEFECTIVE RECOMBINANT ADENOVIRUS" GENE THERAPY, vol. 3, no. 10, October 1996 (1996-10), page 919-926 XP000673471 ISSN: 0969-7128 cited in the application the whole document	1-7, 9-14, 17-20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1999	29/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 99/20730

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 10088 A (UNIV PENNSYLVANIA) 12 March 1998 (1998-03-12) page 4, line 30 -page 14, line 18; claim 26	1,4-6, 9-12, 17-20
A	WO 94 11506 A (ARCH DEV CORP) 26 May 1994 (1994-05-26) the whole document	1,9,14
A	PHILIP R ET AL: "EFFICIENT AND SUSTAINED GENE EXPRESSION IN PRIMARY T LYMPHOCYTES AND PRIMARY AND CULTURED TUMOR CELLS MEDIATED BY ADENO-ASSOCIATED VIRUS PLASMID DNA COMPLEXED TO CATIONIC LIPOSOMES" MOLECULAR AND CELLULAR BIOLOGY, vol. 14, no. 4, April 1994 (1994-04), page 2411-2418 XP000676937 ISSN: 0270-7306 cited in the application the whole document	1,6
A	ZOU Y ET AL., : "CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway" DEVELOPMENT, vol. 124, 1997, pages 793-804, XP000863100 cited in the application the whole document	8,15
A	JEYASEELAN R ET AL., : "A novel cardiac-restricted target for doxorubicin" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 36, 5 September 1997 (1997-09-05), pages 22800-22808, XP002125498 cited in the application page 22803, right-hand column, paragraph 3; figure 4 discussion.	8,15
A	YEH P AND PERRICAUDET M: "Advances in adenoviral vectors: from genetic engineering to their biology" FASEB JOURNAL, vol. 11, no. 8, 1997, page 615-623 XP002086861 ISSN: 0892-6638 cited in the application the whole document	1

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

information on patent family members

Interr. .nal Application No
PCT/US 99/20730

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9613598 A	09-05-1996	US 5856152 A	05-01-1999
		AU 695811 B	20-08-1998
		AU 4405596 A	23-05-1996
		CA 2203808 A	09-05-1996
		EP 0797678 A	01-10-1997
		JP 10507928 T	04-08-1998
		US 5871982 A	16-02-1999
WO 9810088 A	12-03-1998	AU 4183397 A	26-03-1998
		EP 0931158 A	28-07-1999
WO 9411506 A	26-05-1994	AU 694097 B	16-07-1998
		AU 5609394 A	08-06-1994
		CA 2149771 A	26-05-1994
		EP 0668913 A	30-08-1995
		EP 0957172 A	17-11-1999
		JP 8506008 T	02-07-1996

EXHIBIT D

Cardiac Gene Delivery With Cardiopulmonary Bypass

Michael J. Davidson, MD; J. Mark Jones, AFRCS; Sitaram M. Emani, MD; Katrina H. Wilson, MS; James Jaggers, MD; Walter J. Koch, PhD; Carmelo A. Milano, MD

Background—Cardiac gene therapy offers the possibility of enhancing myocardial performance in the compromised heart. However, current gene delivery techniques have limited myocardial transgene expression and pose the risk of extracardiac expression. Isolation of the coronary circulation during cardiac surgery may allow for more efficient and cardiac-selective gene delivery in a clinically relevant model.

Methods and Results—Neonatal piglets (3 kg) underwent a median sternotomy and cardiopulmonary bypass, followed by aortic cross-clamping with 30 minutes of cardioplegic arrest. Adenoviral vectors containing transgenes for either β-galactosidase (adeno-β-gal, n=11) or the human β_2-adrenergic receptor (adeno-β_2-AR, n=15) were administered through the cardioplegia cannula immediately after arrest and were allowed to dwell in the coronary circulation during the cross-clamp period. After 1 week, the animals were killed, and their heart, lungs, and liver were excised and examined for gene expression. Analysis of β-galactosidase staining revealed transmural myocardial gene expression among animals receiving adeno-β-gal. No marker gene expression was detected in liver or lung tissue. β-AR density in the left ventricle after adeno-β_2-AR delivery was 396±85% of levels in control animals ($P<0.01$). Animals receiving adeno-β_2-AR and control animals demonstrated similar β-AR density in both the liver (114±8% versus 100±9%, P=NS) and lung (114±7% versus 100±9%, P=NS). There was no evidence of cardiac inflammation.

Conclusions—By using cardiopulmonary bypass and cardioplegic arrest, intracoronary delivery of adenoviral vectors resulted in efficient myocardial uptake and expression. Undetectable transgene expression in liver or lung tissue suggests cardiac-selective expression. **(*Circulation*. 2001;104:131-133.)**

Key Words: gene therapy ■ cardiopulmonary bypass ■ signal transduction

Cardiac gene transfer of either the human β_2-adrenergic receptor (β_2-AR) or an inhibitor of β-adrenergic receptor kinase (βARKct) enhances cardiac performance.[1,2] Use of such genetic strategies clinically will require a safe method of cardiac gene delivery. The technique that has been used in the laboratory setting involves intracoronary injection of an adenoviral vector with the heart beating. The principal disadvantage of this technique is that the viral vector is rapidly washed out to the systemic circulation and taken up in nontarget organs such as the liver and lung.[1,3] A critical feature of any clinically relevant cardiac gene delivery technique, however, is limiting noncardiac delivery to prevent toxicity.

We hypothesized that cardiopulmonary bypass (CPB) may facilitate cardiac-selective gene transfer using recombinant replication-deficient adenovirus. CPB with aortic cross-clamping and cardioplegic arrest represent the fundamental components of many cardiac surgery procedures and uniquely isolate the coronary circulation. Administration of an adenoviral vector under these conditions maximizes contact time with the myocardium and may reduce systemic delivery, therefore limiting toxicity and offering a clinically relevant delivery system.

Methods

A replication-deficient, first-generation, type V adenovirus with deletions of the E1 and E3 genes was used to construct vectors for the human β_2-AR (adeno-β_2-AR) or β-galactosidase (adeno-β-gal) transgene.[4] Large-scale preparations of these adenoviruses were purified from infected Epstein-Barr nuclear antigen–transfected 293 cells (Invitrogen Corp).[4]

One-week-old piglets (3 kg) received humane care in compliance with the institutional committee on animal research and in accordance with the regulations adopted by the National Institutes of Health. Animals were given ketamine (20 mg/kg IM) just before inhaled isoflurane (1%) anesthesia.[5] A median sternotomy was performed, and after systemic heparinization, CPB was established via an aortic cannula and a right atrial cannula. The CPB circuit consisted of a reservoir, a hollow fiber oxygenator/heat exchanger, and a roller pump. After stabilization, the aorta was cross-clamped and the heart arrested by infusion of cold (4°C), hyperkalemic cardioplegia solution (30 mL/kg) into the aortic root. Animals were randomized to receive either adeno-β_2-AR or adeno-β-gal. Immediately after cardioplegic arrest, 1×10^{11} total viral particles, reconstituted in 8 mL of phosphate-buffered saline (PBS), were injected into

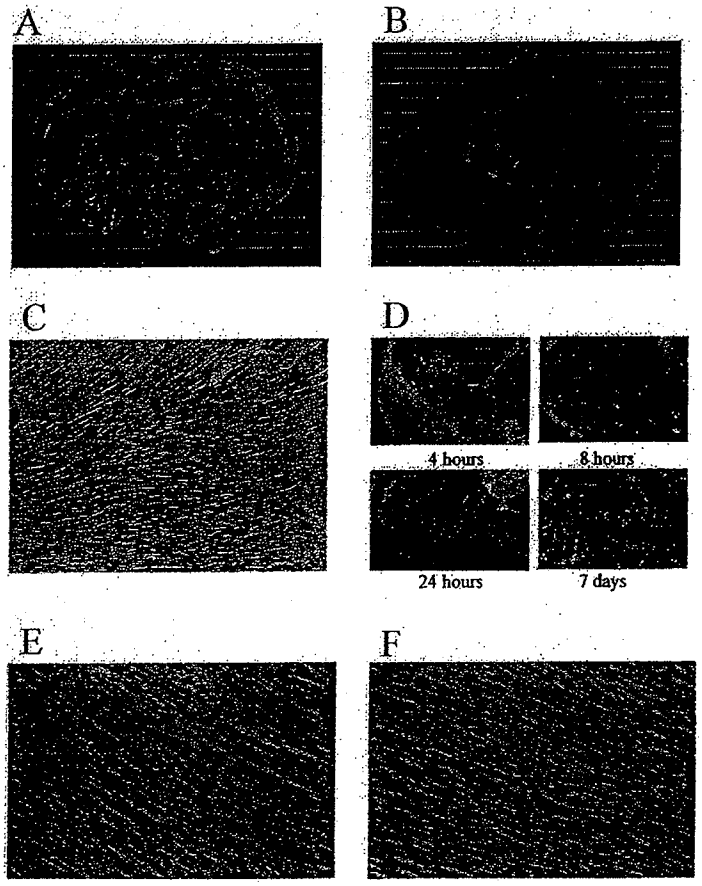
Received March 30, 2001; revision received May 9, 2001; accepted May 11, 2001.

From the Departments of Surgery (M.J.D., J.M.J., S.M.E., J.J., W.J.K., C.A.M.), Medicine, and Biochemistry (K.H.W.), Duke University Medical Center, Durham, NC.

Parts of this work were presented at the October 2000 meeting of the American College of Surgeons, Chicago, Ill.

Correspondence to Carmelo A. Milano, MD, Department of Surgery, Box 3043, Duke University Medical Center, Durham, NC 27710. E-mail david015@mc.duke.edu

Circulation is available at http://www.circulationaha.org



Whole heart mounts and histological sections after gene delivery. A, Whole mount of heart 1 week after adeno-β-gal delivery. B, Whole mount of heart 1 week after adeno-β_2-AR delivery. C, Light micrograph (magnification ×40) of myocardium 1 week after adeno-β-gal delivery, demonstrating X-gal staining of individual myocytes. D, X-gal staining in whole mounts of left ventricle at 4, 8, and 24 hours and 7 days after β-galactosidase transgene delivery. E, Light micrograph (magnification ×40) of myocardium stained with hematoxylin and eosin 1 week after adeno-β_2-AR delivery. F, Light micrograph (magnification ×40) of myocardium stained with hematoxylin and eosin 1 week after PBS delivery.

the aortic root and allowed to dwell in the myocardium. After 30 minutes of cardiac arrest, the cross-clamp was removed and the heart was reperfused. The animals were then weaned off CPB and allowed to recover.

Gene expression was assessed 1 week after delivery. A subset of animals (n=8) was studied at 4, 8, and 24 hours and 14 days after gene delivery to examine the time course of expression. Heart, liver, and lung tissues were either immediately stained with X-gal solution [2 mmol/L $K_4Fe(CN)_6$, 2 mmol/L $K_3Fe(CN)_6$, 2 mmol/L $MgCl_2$, and 0.5 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside] as whole-mount samples or frozen at −80°C, sectioned at 10 μm, and stained in X-gal as previously described.[6] β-AR expression was quantified with radioligand binding assays to determine total β-AR density. Tissue samples were homogenized in lysis buffer (5 mmol/L Tris-HCl [pH 7.4] and 5 mmol/L EDTA), and membrane fractions were extracted. A radioligand binding assay was performed using ^{125}I-cyanopindolol to determine total β-AR density, as previously described.[7]

A subgroup of animals received only PBS during CPB (n=4). Standard hematoxylin and eosin histological sections of these hearts were made and compared with sections from hearts treated with adeno-β_2-AR to assess any inflammatory response.

Data are expressed as mean±SEM and were assessed by Student's *t* test. Significance was assumed at $P<0.05$.

Results

A total of 42 piglets underwent CPB-mediated gene delivery. Of these, 40 survived to the time of study (4 hours to 14 days). Twenty-six piglets were studied for myocardial transgene expression at 1 week. The piglets that received adeno-β_2-AR (n=15) demonstrated no background X-gal staining, whereas those that received adeno-β-gal (n=11) had transmural staining in all chambers (Figure, A and B). Micrographic sections of the myocardium of animals receiving β-galactosidase demonstrated staining of individual myocytes, consistent with transgene expression (Figure, C). There was no β-galactosidase expression in the liver or lung.

Animals treated with adeno-β_2-AR exhibited a left ventricular β-AR density ≈4-fold higher than those receiving marker transgene ($P<0.01$; Table). The right ventricular β-AR density was 1.6-fold higher than that of control animals, demonstrating lower but significant transgene expression in this chamber ($P=0.01$). β-AR density was not different in the liver and lung between adeno-β_2-AR and adeno-β-gal–treated animals (Table).

In addition, gene expression was studied at varying intervals from time of delivery (Figure, D). β-Galactosidase

β-AR Density in Treated and Control Piglets

Tissue	Adeno-β_2-AR (n=15)	Adeno-β-gal (n=11)
Left ventricle	396±85%*	100±7% (94.4 fmol/mg)
Right ventricle	164±19%*	100±9% (101.0 fmol/mg)
Liver	114±7%	100±9% (77.4 fmol/mg)
Lung	114±8%	100±11% (137.4 fmol/mg)

Values are mean±SEM and are expressed as percent of control. All studies were conducted 1 week after gene delivery.

*$P<0.05$ vs control.

expression was first detected 8 hours after gene delivery. Expression at 8 hours was transmural and comparable to that seen at 24 hours and at 1 week. At 2 weeks, an additional 4 animals treated with adeno-β_2-AR had increased left ventricular (275±126 fmol/mg) and right ventricular (181±31 fmol/mg) β-AR density.

Hematoxylin and eosin micrographs of hearts 1 week after delivery of adeno-β_2-AR or PBS (n=4) are shown in the Figure (panels E and F, respectively). There was no evidence of an inflammatory response in either group.

Discussion

This study demonstrates the feasibility of myocardial gene delivery during CPB and cold hyperkalemic cardioplegic arrest. This protocol simulates conventional cardiac surgery and tests the effectiveness and potential advantages of gene transfer during cardiac surgery. Unlike prior attempts at intracoronary gene transfer, CPB-mediated gene therapy seems to limit extracardiac gene expression. Our laboratory has previously found high levels of gene expression in the liver and lung after non-CPB intracoronary delivery.[1] When delivered to the beating heart, the intracoronary vector is rapidly washed out of the heart and delivered systemically.

Because the coronary circulation is uniquely isolated during CPB, gene delivery to the myocardium may be improved relative to injection into the coronary circulation with the heart beating. By using CPB and cardioplegic arrest, the virus is allowed to dwell in the coronary circulation for 30 minutes. At the end of this time, in contrast to beating-heart delivery, a higher percentage of viral particles may be taken up by myocytes or be inactivated. Furthermore, any remaining viable virus is ultimately washed out of the coronary circulation via the coronary sinus and returned to the CPB apparatus. Because the CPB circuit has a high surface area for potential virus-binding, particularly at the membrane oxygenator, the remaining viable virus may become bound. Indeed, Marshall et al[8] demonstrated that the replication-deficient adenoviral vectors commonly used for gene delivery are rapidly inactivated on exposure to nonbiological surfaces such as polycarbonate, cardiac catheters, and syringes.

This approach may have multiple applications to clinical cardiac surgery. Such genetic treatments might support end-stage heart failure patients in a manner similar to left ventricular assist devices, as a bridge of support until heart transplantation. It may also provide support for high-risk patients with severely reduced ventricular function undergoing revascularization or valve replacement procedures. Indeed, impairment of the myocardial β-AR system during cardiac surgery has been documented, including receptor desensitization with reduced adenylyl cyclase response, possibly due to increased βARK1 activity.[9,10] This method of gene therapy would achieve transgene expression during the first postoperative day and continue for ≈2 to 3 weeks. This time course would correlate with the early postoperative period during which inotropic support is most important. These studies also raise interest in the possibility of gene therapy with retrograde cardioplegia or with percutaneous methods of CPB, such as Heartport.

This study represents the first use of CPB for global myocardial gene delivery. Moreover, it demonstrates the feasibility of intracoronary gene delivery in the pig, whose heart is similar to humans. The study is limited insofar as the subjects were healthy neonatal piglets. Further work is needed to characterize the effectiveness of this technique in adult animals and those with ventricular dysfunction. In addition, current efforts are directed at demonstrating the biochemical and hemodynamic consequences of gene delivery using functional transgenes.

Acknowledgments

This work was supported in part by National Institute of Health grants HL61690 (to W.J.K.) and HL56205 (to W.J.K.) and by National Research Service Award 5 F32 HL10179 (to M.J.D.). The authors thank George Quick, Ronnie Johnson, and Kurt Campbell for their invaluable assistance in the animal setup and use of CPB. We also thank Robert J. Lefkowitz, who was instrumental in initiating gene therapy efforts with β_2-AR and who provided much of the adenoviral vector for these studies.

References

1. Maurice J, Hata J, Shah A, et al. Enhancement of cardiac function after adenoviral-mediated in vivo intracoronary β-2-adrenergic receptor gene delivery. *J Clin Invest*. 1999;104:21–29.
2. White D, Hata J, Shah A, et al. Preservation of myocardial β-adrenergic receptor signaling delays the development of heart failure after myocardial infarction. *Proc Natl Acad Sci USA*. 2000;97:5428–5433.
3. Hajjar R, Schmid U, Matsui T, et al. Modulation of ventricular function through gene transfer in vivo. *Proc Natl Acad Sci USA*. 1998;95:5251–5256.
4. Akhter S, Skaer C, Kypson A, et al. Restoration of β-adrenergic signaling in failing cardiac ventricular myocytes via adenoviral-mediated gene transfer. *Proc Natl Acad Sci USA*. 1997;94:12100–12105.
5. Lodge A, Chai P, Daggett C, et al. Methylprednisolone reduces the inflammatory response to cardiopulmonary bypass in neonatal piglets: timing of dose is important. *J Thorac Cardiovasc Surg*. 1999;117:515–522.
6. Kypson A, Peppel K, Akhter S, et al. Ex-vivo adenoviral-mediated gene transfer to the transplanted adult rat heart. *J Thorac Cardiovasc Surg*. 1998;115:623–630.
7. Koch W, Rockman H, Samama P, et al. Cardiac function in mice overexpressing the Beta-adrenergic receptor kinase or a BARK inhibitor. *Science*. 1995;268:1350–1353.
8. Marshall D, Palasis M, Lepore J, et al. Biocompatibility of cardiovascular gene delivery catheters with adenovirus vectors: an important determinant of the efficiency of cardiovascular gene transfer. *Mol Ther*. 2000;1:423–429.
9. Schwinn D, Leone B, Spahn D, et al. Desensitization of myocardial β-adrenergic receptors during cardiopulmonary bypass: evidence for early uncoupling and late downregulation. *Circulation*. 1991;84:2559–2567.
10. Sun L, Pantuck C, Morelli J, et al. Perioperative lymphocyte adenylyl cyclase function in pediatric cardiac surgical patients. *Crit Care Med*. 1996;24:1654–1659.

EXHIBIT E

0013-7227/01/$03.00/0
Endocrinology

Vol. 142, No. 1
Printed in U.S.A.

Type 2 Iodothyronine Deiodinase Transgene Expression in the Mouse Heart Causes Cardiac-Specific Thyrotoxicosis*

JANUSZ PACHUCKI, JAMES HOPKINS, ROBIN PEETERS†, HELEN TU, SUZY D. CARVALHO, HELEN KAULBACH, E. DALE ABEL, FREDERIC E. WONDISFORD, JOANNE S. INGWALL, AND P. REED LARSEN

Thyroid Division (J.P., R.P., H.T., S.D.C., P.R.L.), Brigham and Women's Hospital, Harvard Institute of Medicine, Boston, Massachusetts 02115; Department of Internal Medicine and Endocrinology (J.P.), University Medical School of Warsaw, 02–097 Warsaw, Poland; Nuclear Magnetic Resonance Laboratory for Physiological Chemistry (J.H., J.S.I.), Brigham and Women's Hospital, Boston, Massachusetts; Thyroid Unit (H.K., E.D.A., F.E.W.), Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

ABSTRACT

Type 2 iodothyronine deiodinase (D_2) catalyzes intracellular 3, 5, 3′ triiodothyronine (T_3) production from thyroxine (T_4), and its messenger RNA mRNA is highly expressed in human, but not rodent, myocardium. The goal of this study was to identify the effects of D_2 expression in the mouse myocardium on cardiac function and gene expression. We prepared transgenic (TG) mice in which human D_2 expression was driven by the α-MHC promoter. Despite high myocardial D_2 activity, myocardial T_3 was, at most, minimally increased in TG myocardium. Although, plasma T_3 and T_4, growth rate as well as the heart weight was not affected by TG expression, there was a significant increase in heart rate of the isolated perfused hearts, from 284 ±12 to 350 ± 7 beats/min. This was accompanied by an increase in pacemaker channel (HCN2) but not α-MHC or SERCA II messenger RNA levels. Biochemical studies and ^{31}P-NMR spectroscopy showed significantly lower levels of phosphocreatine and creatine in TG hearts. These results suggest that even mild chronic myocardial thyrotoxicosis, such as may occur in human hyperthyroidism, can cause tachycardia and associated changes in high energy phosphate compounds independent of an increase in SERCA II and α-MHC. (*Endocrinology* **142:** 13–20, 2001)

THE HEART is one of the most sensitive organs to increases in thyroid hormone. Patients with hyperthyroidism virtually always have tachycardia and an increased rate of myocardial contraction (1). This is attributed to both intrinsic and extrinsic effects of the excess hormone (2, 3). Based on animal models, the intrinsic effects of thyroid hormone on the heart are thought to be due to changes in the expression of certain genes including genes for myosin heavy chains (MHC), sarcoplasmic reticulum calcium ATPase (SERCA II), and hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2) (4–7). The extrinsic effects are those arising from the necessity for a myocardial response to the increase in oxygen demand induced by the hyperthyroid state (2).

Thyroxine (T_4) is a tetra-iodinated iodothyronine prohormone, which must be mono-deiodinated in the outer ring to T_3 to be activated (1, 8). In mammals, there are two isoenzymes that can catalyze this conversion, the types 1 (D1) and 2 (D2) 5′ iodothyronine deiodinases (8). In rat tissues expressing D2, a substantial portion of the nuclear receptor-bound T_3 is provided by the intracellular conversion of T_4 to T_3 by this isoenzyme (9). This enzyme is a critical component of the homeostatic mechanism for maintaining the tissue T_3 under a variety of stresses because it can increase the efficiency of T_4 activation when T_4 production is reduced as in iodine deficiency (10).

The coding sequence and 3′ untranslated region of the human type 2 deiodinase have been recently identified (11–13). The messenger RNA (mRNA) is highly expressed not only in the human brain and pituitary, as it is in the rat and mouse, but also in myocardium and skeletal muscle, which is not the case in the rodent (12–14). The expression of D2 in the myocardium raises the possibility that, in humans, this tissue can respond not only to changes in plasma T_3, but also to those in T_4. Thus, the human heart may resemble the pituitary and brain with respect to sources of intracellular T_3. This could contribute to the sensitivity of pulse rate to minimal increases in circulating T_4. The contribution of the T_3 generated by the action of D2 to total myocardial T_3 in the human heart under normal or pathological conditions remains to be determined. However, it seems likely that with respect to the potential for the intracellular T_4 to contribute to intracellular T_3 in this organ, rodents are not a faithful model of the human situation.

Studies of the effects of thyroid hormone on the myocar-

Received July 5, 2000.
Address all correspondence and requests for reprints to: P. Reed Larsen, M.D., FACP, FRCP, Chief, Thyroid Division, Brigham and Women's Hospital, 560 Harvard Institute of Medicine, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115. E-mail: larsen@rascal.med.harvard.edu.
* This work was supported by grants from the NIH [DK-36256 (to P.R.L.); DK-49126 and DK-53036 (to F.E.W.) and Specialized Center of Research Grant HL-52320 (to J.S.I.)].

† Supported by a Student's fellowship from Dr. Saal van Zwanenbergstichting, Stichting Bekker-la Bastide-Fonds and Stichting Dr. Hendrik Muller's Vaderlandsch Fonds from Netherlands.

dium of experimental animals are often performed by giving exogenous T_4 or T_3 to hypothyroid rats, generally in large excess, for relatively short time periods. Although this has allowed the identification of number of T_3-responsive genes, including α- and β-MHC, SERCA II, HCN2 as well as inducing acute changes in the cardiac physiology of the hyperthyroid animal, it does not faithfully replicate the pathophysiology of human hyperthyroidism. Clinical hyperthyroidism is typically present for at least 6 months in a progressively more symptomatic form before coming to medical attention (1). Biochemically, *e.g.* in terms of suppression of serum TSH, it has likely to have been present for an even longer period. Usually, there is only a 2- to 3-fold increase in serum T_4 (1). For these reasons, the animal experiments do not accurately replicate human hyperthyroidism.

To provide a model that might better reflect events in the hyperthyroid human myocardium with respect to the sources of T_3, we have prepared transgenic (TG) mice in which human D2 is driven by the mouse α-MHC promoter and, therefore, expressed at high levels in the myocardium. The mice are systemically euthyroid but have some, but not all, physiological, biochemical, and molecular changes in the heart consistent with thyrotoxicosis. Perfused hearts were tachycardic, had an increase in rate pressure product and a decrease in phosphocreatine (PCr) without any changes in creatine kinase activity. The HCN2 mRNA was modestly increased, but no significant changes were found in the expression of α-MHC or SERCA II genes.

Materials and Methods

Animals

All aspects of animal care and experimentation performed in this study were approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center and the Brigham and Women's Hospital. Animals were maintained on a 12-h light/12-h dark schedule (light on at 0600 h) and fed laboratory chow and water *at libitum* if not otherwise indicated. Experimental hypothyroidism was introduced by a low iodine, PTU-containing diet (Remington diet, Harlan Teklad, Madison, WI). The studies were performed using mice 2–8 months old.

Generation and screening of transgenic animals

The coding region of the human D2 complementary DNA (cDNA) (Genethon clone supplied by Drs. St. Germain and Galton, Dartmouth Medical Center, Lebanon, NH) and the selenocysteine insertion sequence (SECIS) of rat selenoprotein P (SelP), as a 1.9-kb fragment with the potential poly A site, were subcloned between the mouse α-MHC 5′ flanking region and the additional polyadenylation sequence of human GH (vector provided by Dr. Jeffrey Robbins, Division of Molecular Cardiovascular Biology, University of Cincinnati Medical Center, Cincinnati, OH) to form plasmid pHT1402 (Fig. 1). The entire 8.4-kb transgene was released from the plasmid pHT1402 by *Bam*HI digestion. Approximately 4 ng of the gel purified transgene were microinjected into each male pronuclei of 1-day-old mouse zygotes of the inbred strain FVB/C57, and these were reimplanted into the uteri of pseudopregnant foster mice at The Beth Israel Transgenic Facility. Litters were obtained after 21 days. Between age 14 and 18 days, preweaned mice were identified by gender, marked by earlobe punching, and approximately 10 mm of tail tip was removed for genotyping. Genomic DNA was generated by overnight digestion with proteinase K and SDS. After high salt precipitation in the presence of SDS, the supernatant was phenol/chloroform extracted and DNA precipitated by ethanol. Genomic DNA (10 μg) was digested with *Xba*I (20 U/μg of DNA) subjected to electrophoresis through the 1% agarose gel and transferred to GeneScreen Plus nylon membranes (NEN Life Science Products, Boston, MA). Hybridization was performed using a 0.3 kb *Xba*I; *Acc*I fragment of rat D2 cDNA, which is virtually identical in sequence to the mouse and human D2 genes (13, 15). The rat D2 cDNA was kindly provided by Drs. St. Germain and Galton (Dartmouth Medical Center, Lebanon, NH). Two transgenic lines were identified and expanded. Littermates served as controls unless indicated.

Serum T_3 and T_4 measurements

Blood was collected after decapitation, centrifuged, sera separated, and stored at −20 C until used for assay. RIA for T_4 and T_3 was done in duplicate, using 10 μl and 50 μl of each mouse sera respectively, as described earlier (16). Standards were prepared in rat serum, which had been depleted of endogenous T_3 and T_4 by charcoal adsorption. The limit of detection was approximately 2 pg of T_4 and approximately 1 pg of T_3 per tube.

Determination of myocardial T_3 concentration

Animals were anesthetized by carbon dioxide and decapitated. After wide opening of chest cavity, hearts (including both atria and ventricle) were rapidly dissected from the great vessels, divided in half, rinsed in ice-cold PBS, and frozen in liquid nitrogen. Half of each heart, about 50 mg, was used for measurement of T_3 content. Each tissue was homogenized in 1 ml of methanol using a Brinkmann Instruments, Inc. (Westbury, NY) homogenizer. For protein measurement, a 50-μl aliquot of each homogenate was solubilized by adding 10 μl of 1 M NaOH. The solubilized protein was diluted in water and concentration of protein was measured using Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay kit. To assess the recovery of T_3, approximately 500 cpm of the high specific activity [^{125}I]-T_3 (NEN Life Science Products, Boston, MA) were added to the rest of each homogenate and counted. Homogenates were then spun for 20 min at 5,000 × *g* and the supernatant mixed with 2 ml of chloroform. Thyroid hormone was extracted into aqueous solution by two successive 0.5 ml aliquots of 0.4 M NH_4OH. The supernatants were pooled after centrifugation for 20 min at 5,000 × *g*. Any possible traces of chloroform in pooled supernatants were removed by adding 1 ml of ethyl ether and gravity separation. Samples were then evaporated in a lyophilizer (Freezemobile 12 SL, The Virtis Co., Gardiner, NY) and redissolved in 400 μl of 0.01 M NaOH. Each sample was again counted to determine the T_3 recovery which ranged from 60–75%. Duplicate samples of solubilized T_3 were assayed in sodium salicylate/0.2 M glycine acetate buffer, pH 8.6, using specific and sensitive rabbit polyclonal T_3 antibodies (17, 18). Standards were prepared in 0.01 M NaOH and ranged from 0.5–16 pg of T_3/tube. Additionally, the same aliquot of each sample and standard was used to determine nonspecific binding of [^{125}I] T_3 in the absence of T_3 antibodies. This did not differ between standards and samples (18). Linearity of measurement was confirmed by assay of four serial 2-fold dilutions of T_3 extracts from rat heart and liver.

Perfusion protocol

Mice of both genders from both lines were heparinized (5000 U/kg BW, administered ip) 10–15 min before cervical dislocation. Their hearts were excised and immediately arrested by placing in ice-cold perfusion buffer. After cannulation of the aorta, each heart was perfused by the Langendorff method at constant pressure of 80 mmHg and at 37.5 C with modified Krebs Henseleit bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM Na_2EDTA, 25 mM $NaHCO_3$, 10 mM glucose, and 0.5 mM pyruvate). All buffers were gassed with 95% O_2/5% CO_2 to give a pH of 7.4 at 37 C.

Cardiac function was recorded as the rate pressure product (RPP), the product of heart rate and left ventricular developed pressure (LVDP), using a water-filled polyethylene balloon in the left ventricle. The size of the balloon was carefully matched to the size of the ventricle (19). The balloon was connected via a water-filled tube to a pressure transducer (Stratham P23Db, Gould, Oxnard, CA) attached to a MacLab (ADInstruments, Milford, MA) analog digital converter, sampling at 200 samples/sec. The balloon was inflated to give an end diastolic pressure (EDP) of approximately 8 mmHg. Intraventricular pressure development was prevented by

inserting a short piece of polyethylene (PE10) tubing through the apex of the left ventricle.

Hearts were placed in a 10 mm NMR tube, and the effluent from the heart was suctioned from above. The effluent flow rate was measured in a volumetric container, which allowed the coronary flow rates to be calculated. The perfusion system was then placed into the magnet at the correct height to give 80 mmHg pressure at the level of the heart. The temperature of the system was maintained at 37.5 C by external heating of the NMR tube with warm air and by keeping all perfusion buffers in water jacketed containers (20). Each group of hearts was subjected to a stabilization period of 30 min, during which the probes were tuned and the magnet shimmed. Following that, two 8 min spectra were acquired. At the end of each experiment the hearts were blotted and weighed, and stored at −80 C.

^{31}P NMR spectroscopy and data analysis

Spectra were acquired using a GE-400 wide bore Omega NMR spectrometer (GE, Fremont, CA) operating at the ^{31}P resonance frequency of 161.94 MHz. A 10-mm glass NMR tube (Wilmad, Buena, NJ) containing the isolated heart preparation was inserted into a custom built $^{1}H/^{31}P$ double-tuned probe (Morris Instruments, Ontario Canada) situated in the center of a 9.4 T superconducting magnet. The spectra were acquired as described previously (20, 21). Quantification of ATP, PCr and Pi concentrations from spectral peak areas was achieved using biochemically determined ATP concentration in a separate group of hearts which were freeze-clamped after the same period of perfusion. The ATP resonance area (average of α and γ phosphate areas) divided by the wet weight (mgww) of each heart was used to convert the resonance areas of the other phosphorus containing metabolites using their saturation factors previously determined in our laboratory for PCr (1.2) and inorganic phosphate, Pi (1.15), relative to ATP. Myocardial pH was estimated using the chemical shifts of the Pi peak relative to the PCr peak using titration curves determined previously in our laboratory (pH = ppm·0.724 + 3.5455 were ppm reflects the chemical shift between Pi and PCr). Cytosolic free ADP concentration was calculated using the equilibrium constant of the CK reaction ($K_{eqm} = 1.99 \times 10^{9}$ M^{-1}) and using metabolite values obtained by NMR spectroscopy and biochemical analysis (22, 23).

Biochemical analysis

A separate group of hearts was freeze-clamped after the same period of perfusion. These were stored at −80 C, and used for biochemical determination of ATP, PCr, Creatine (Cr), glucose-6-phosphate (G6P). ATP, PCr, Cr, glycogen, and G6P were extracted in 6% perchloric acid and assayed using spectrophotometric techniques as described (24). ATP, PCr, Cr, and G6P results were calculated in mmol/mg of protein and expressed in mM concentration using the conversion factor 0.17 (protein/wet weight ratio) and factor 0.48 (water/wet weight ratio). For glycogen measurements the wet/dry weight ratio of each heart was determined, and results expressed as μmol/g dry wt (μmol glucosyl units/g dry wt for glycogen). Creatine kinase activity (CK V_{max}) and the amount of this activity attributable to each isoenzyme of CK as well as adenosine kinase (AK) activity were measured using methods previously described (25). The cardiac tissue was homogenized for 10 sec at 4 C in potassium phosphate buffer containing 1 mmol/liter EDTA and 1 mmol/liter β-mercaptoethanol, pH 7.4 (final concentration of 5 mg tissue/ml). Triton X-100 was then added to the homogenate at a final concentration of 0.1%. The CK activity was measured in tissue homogenates at 30 C (25). CK activities were measured in units of IU per mg protein and converted to mM/sec using the measured concentrations of cardiac protein, assayed in the samples before the addition of Triton X-100 using the Lowry method (26). All values are expressed as mM/sec at 37 C (the results were multiplied by the factor 1.8 to convert from 30–37 C). The percent of total CK activity attributable to each isoenzyme was measured using a Helena Cardio-Rep CK isoenzyme analyzer (Beaumont, TX) (25).

Deiodinase assays

Tissues were homogenized on ice in buffer containing 1 × PE (0.1 M potassium phosphate and 1 mM EDTA), 250 mM sucrose and 10 mM DTT (pH 6.9). D2 assays were performed in the presence of 1 nM T_4 with or without 1 mM PTU and/or 100 nM T_3, as described earlier (27).

Isolation and analysis of RNA

RNA was extracted from the tissue using TriZol reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol and RNA concentration was estimated from the A260 value. Northern analysis was performed using of 10 μg total RNA by standard methods as described earlier (7). A mouse HCN2 cDNA fragment (~0.5 kb) was made by RT-PCR from euthyroid mouse cortex RNA (7). Specific α-MHC oligonucleotide was a gift of Dr. W. H. Dillmann (Department of Medicine, Division of Endocrinology and Metabolism, University of California San Diego, La Jolla, CA) and β-MHC oligonucleotide was obtained from Life Technologies, Inc. (Rockville, MD). Both oligonucleotides were designed from the nonhomologous 3′ regions of the mouse myosin heavy chain cDNAs and have been described earlier (28). Mouse β-actin cDNA was a gift of Dr. B. M. Spiegelman (Dana Farber Cancer Institute, Boston, MA). Rat cyclophilin cDNA was a gift of Dr. G. Adler and W. Chin (Brigham and Women's Hospital, Boston, MA). Labeling of α- and β-MHC probes was performed by 5′ end-labeling method using T_4-polynucleotide kinase from New England Biolabs, Inc. (Beverly, MA) and [γ^{32}P] ATP from NEN Life Science Products (Boston, MA). The remaining probes were radiolabeled using standard random nanomer method and [α^{32}P] dCTP.

Statistical analysis

All results are expressed as means ± SEM. Statistical analysis was done using SPSS, Inc. program version 8 (Chicago, IL). WT and TG mice were compared using ANOVA or Student's t test.

Results

Transgene expression

The transgene construct, pHT1402 is shown in Fig. 1. Southern blotting identified a 3-kb *Xba*I fragment of the mouse type 2 deiodinase (*dio2*) gene as well as a 1.35-kb band of the human D2 transgene both containing sequences ho-

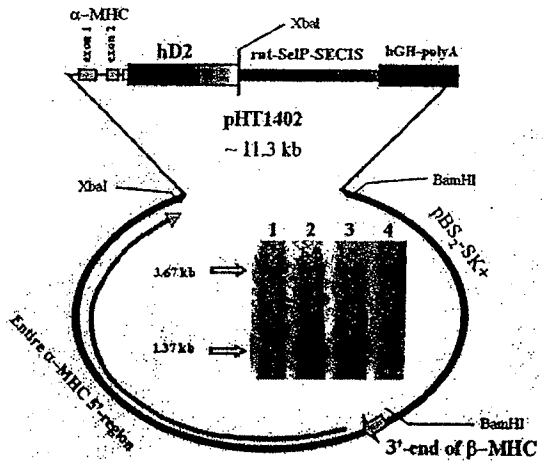


FIG. 1. The human type 2 iodothyronine deiodinase (hD_2) transgene construct (pHT1402) in the pBS2-SK+ vector. The entire region 5′ to the translation initiation codon in exon 3 of the mouse α-MHC gene was used to drive the tissue-specific expression of hD_2. The hD_2 coding sequence was flanked at the 3′ position by the SECIS element of rat SelP to allow for insertion of selenocysteine. *Bam*HI was used to excise the transgene for oocyte injection. Mouse genomic DNA was digested with *Xba*I. TG mouse DNA produced a 1.37-kb fragment of the transgene (lanes 2 and 3 on the inserted picture) and a 3.67 kb native mouse D_2 band (lanes 1 to 4), which hybridized with an *Xba*I, *Acc*I coding fragment of rat D_2 cDNA.

mologous to the fragment of the second exon of the rat *dio2* gene used as a probe (Fig. 1). Two founders, one female and one male, were selected and bred. Based on inheritance and the signal intensity, line 1 had about 50 copies of transgene in the same chromosome and line 2, about 50 copies of transgene in at least two different chromosomes. Neither line exhibited any phenotypic developmental abnormalities nor showed any increased mortality. The growth rate, body weight (BW) and heart to body weight ratio (H/BW) were not statistically different between WT and TG mice (estimated marginal means for 14 week old mice were: 24.3 g *vs.* 24.0 g, $P = 0.87$ for BW and 4.32 mg/g *vs.* 4.14 mg/g, $P = 0.59$ for H/BW ratio). Both WT and TG males had slightly larger body weight then age-matched females (estimated marginal means for 14-week-old mice were 26.8 for males and 21.7 for females, $P = 0.16$, WT plus TG combined).

Northern blot analysis in both lines confirmed a high level of transgene expression in the heart and lungs, the organs where the α-MHC gene is normally expressed (29). The D2 activity in the TG heart was almost 100 and 1000 times higher, respectively, than in the pituitary gland or in the cortex where D2, but not the α-MHC gene, is normally expressed. The kinetic studies of outer ring T_4 deiodination showed, typical for D2, Km of about 1 nM, and lack of PTU inhibition. There were no age (tested by the regression analysis, R = 0.27, $P = 0.32$) or gender-dependent significant differences in transgene expression (males 58.7 ± 8, and females 81 ± 8 pmol of I released/mg·h, $P = 0.09$, n = 11 for males and n = 5 for females). Based on a 2 h assay with 300 μg of myocardial protein, wild-type mouse heart did not contain D2 activity and no mD2 mRNA was found by Northern analysis.

Thyroid hormone concentrations in myocardium and blood

The myocardial T_3 concentration was 12.8 ± 0.9 ng of T_3/g of protein in TG (n = 17) and 11.2 ± 0.8 ng of T_3/g of protein in WT mice, $P = 0.2$ (n = 15). There was a tendency for the myocardial T_3 to be higher in TG than in WT males, but this was not statistically significant (14.8 *vs.* 11.4 ng of T_3/g of protein, $P = 0.09$). There were no significant differences in myocardial T_3 content between lines 1 and 2 (11.3 and 13.6 ng of T_3/g of protein, $P = 0.31$). Serum T_3 and T_4 concentrations were not different between TG and littermate WT mice indicating that there was no significant increase in systemic T_3 production (0.48 *vs.* 0.53 ng/ml, $P = 0.15$, n = 48 and 27.7 *vs.* 27.7 ng/ml, $P = 0.96$, n = 49 for T_3 and T_4, respectively). There were no gender differences in serum T_3 and T_4 concentrations (0.50 *vs.* 0.51 ng/ml, $P = 0.63$ and 28.3 *vs.* 27.1 ng/ml, $P = 0.91$, males *vs.* females, respectively).

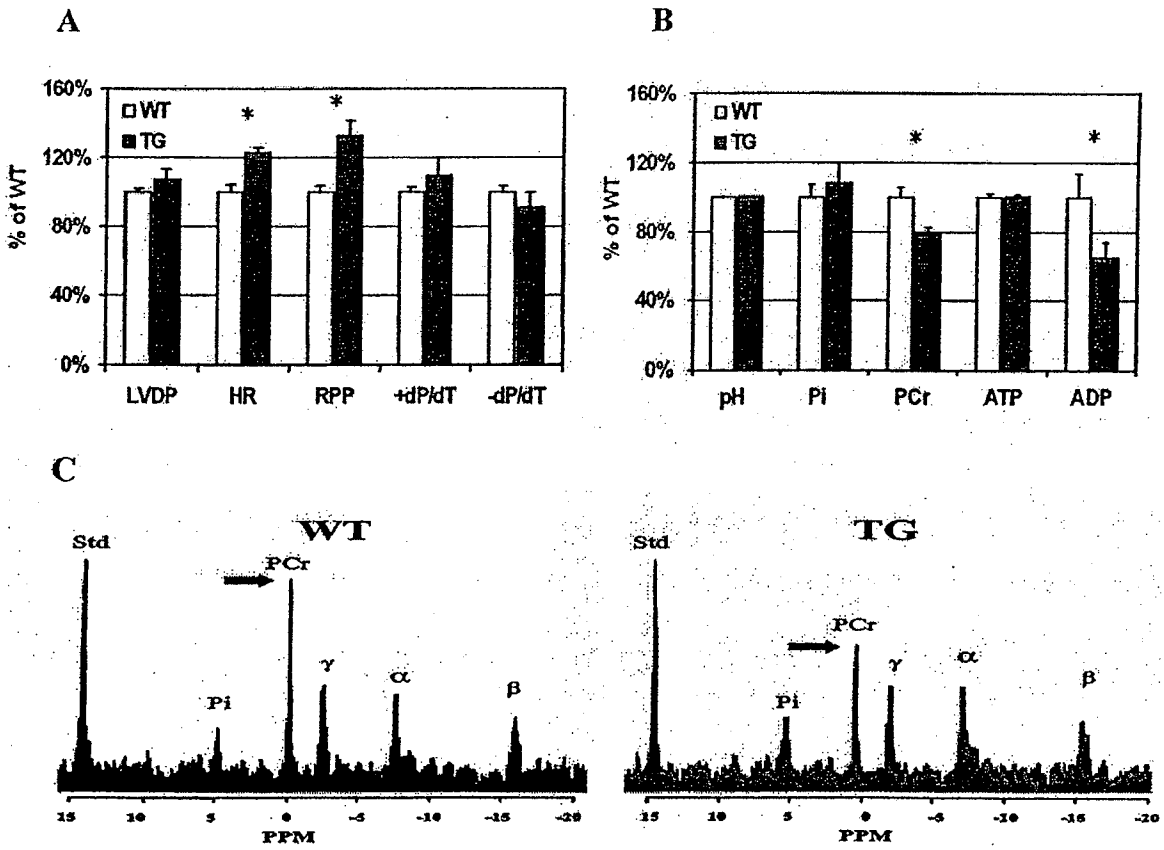


FIG. 2. Physiological parameters (A) and results of ^{31}P NMR spectroscopy (B) during isolated perfusion of mouse hearts (mean and SEM for the 8 WT and 8 TG hearts expressed as % of the mean WT values, * $P < 0.05$). LVDP, Left ventricular developed pressure; HR, heart rate; RPP, rate pressure product, +dP/dT, rate of systolic pressure rise; −dP/dT, rate of diastolic pressure fall; Pi, inorganic phosphate, PCr, phosphocreatine; TP, total phosphate; *, $P < 0.05$. In WT hearts mean LVDP = 91 mm of Hg, HR = 284 beats/min, RPP = 25842 mm of Hg·beats/min. C, Representative ^{31}P NMR spectra of a WT and a TG heart.

Myocardial function and high energy phosphate compounds

Myocardial performance parameters are shown in Fig. 2A. The perfused TG and WT hearts (8 littermate and 4 inbred FVB/C57 wild-type mice matched for age and gender) were the same size. Transgenic hearts exhibited greater basal cardiac function as reflected in a higher basal heart rate and rate pressure product, although 2/3 of this effect was due to the increase in heart rate (Fig. 2A). Therefore contractile function (expressed as RPP/g) in the TG hearts was increased about 25% compared with wild-type hearts ($2.87 \pm 0.23 \times 10^5$ mmHg/min/gww *vs.* $2.18 \pm 0.12 \times 10^5$ mmHg/min/gww; $P = 0.014$). There was no difference in +dP/dt between groups (TG mice: 4222 ± 381 mmHg/sec *vs.* WT mice: 3843 ± 121 mmHg/sec; $P = 0.08$). Similarly, there was no increase in the relaxation rate in the transgenic hearts at baseline (TG mice: −2327 ± 228 mmHg/sec *vs.* WT mice: −2550 ± 95 mmHg/sec; $P = 0.18$).

We also assessed the consequences of mild chronic myocardial thyrotoxicosis by ^{31}P NMR spectroscopy and direct biochemical measurements. The spectral analysis showed a decrease in PCr and PCr/ATP and calculated ADP values in TG hearts (Fig. 2, B and 2C). Biochemical measurements confirmed lower PCr as well as showing depressed total creatine concentration in TG hearts (Table 1). The levels of ATP, Pi and free energy of hydrolysis of ATP were unchanged in the TG hearts. The calculated intracellular pH was also unchanged. There was no difference in the myocardial levels of glycogen or glucose-6-phosphate (G-6-P). CK activity in the transgenic heart was unchanged (51.0 ± 7.0 mM/sec in WT and 48.9 ± 3.0 mM/sec in TG mice, $P = 0.79$, n = 4 for each group) and there were no differences in the activity of CK isoenzymes (Table 1).

Effects of myocardial thyrotoxicosis on thyroid hormone responsive genes

We evaluated the effect of myocardial D2 expression on the mRNA concentrations of α-MHC, β-MHC, SERCA II, and HCN2 in the ventricular myocardium by Northern analysis (Fig. 3). The mRNA for the HCN2 gene has recently been shown to increase in response to thyroid hormone in rats (7).

TABLE 1. Effect of transgene on concentration of various relevant substrates or enzymes

Substance assayed[a]	TG (mean ± SEM)	WT (mean ± SEM)	p[b]
Glycogen (μmol/g of dw)	81.7 ± 7.7	73.5 ± 10.8	0.55
G6P (mM)	0.21 ± 0.05	0.53 ± 0.19	0.15
ATP (mM)	10.8 ± 0.4	10.8 ± 0.6	0.97
PCr (mM)	9.8 ± 0.3	15.7 ± 1.6	0.01
Cr (mM)	19.1 ± 1.0	28.9 ± 1.1	<0.01
Mean CK (mM/sec)	48.9 ± 3.0	51.0 ± 7.0	0.79
MB CK (mM/sec)	1.98 ± 0.09	2.16 ± 0.34	0.61
BB CK (mM/sec)	0.84 ± 0.17	0.64 ± 0.08	0.33
MM CK (mM/sec)	27.9 ± 1.8	29.1 ± 4.0	0.78
Mitochondrial CK (mM/sec)	18.2 ± 1.3	19.1 ± 2.6	0.78
AK (mM/sec)	6.8 ± 0.7	5.3 ± 0.9	0.23

[a] All hearts (n = 4 for each group) were perfused for 30 min before freezing. G6P, Glucose 6-phosphate; PCr, phosphocreatine; Cr, creatine; CK, total creatine kinase; MB, BB, MM, cardiac, brain, skeletal muscle specific creatine kinase isoenzyme; AK, adenosine kinase.
[b] Statistical analysis was done by *t* test.

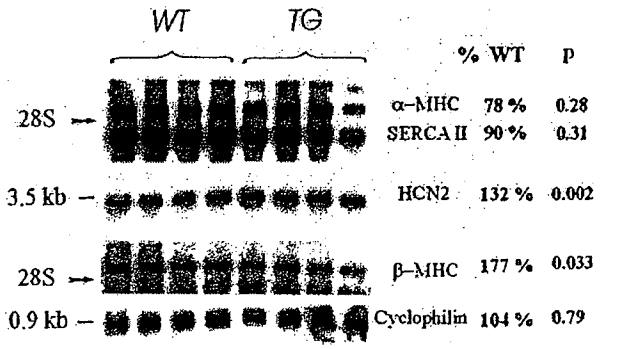


FIG. 3. Representative Northern blots of 4 WT and 4 TG myocardial total RNA probed for various thyroid hormone responsive genes and cyclophilin. The statistical analysis was performed for 12 WT and 12 TG hearts. Only the differences in HCN2 and β-MHC were statistically significant. In all hearts the β-MHC mRNA was much less abundant then α-MHC mRNA such that blots were exposed for 8 days for β-MHC, whereas only 8 h for α-MHC.

We also found this was increased in TG mice ($P < 0.05$, n = 12, Fig. 3). Surprisingly, α-MHC and SERCA II mRNAs were not altered (Fig. 3). The β-MHC mRNA was significantly increased in TG hearts, although in the euthyroid state both WT and TG myocardium expressed almost exclusively α-MHC mRNA (Fig. 3).

Thyroid hormone regulation of the α-MHC-D_2 transgene

The expression of D2 mRNA was driven by the α-MHC promoter, which is positively regulated by thyroid hormone (30). However, because the endogenous α-MHC mRNA was not affected by the rather minimal increase in myocardial T_3, it was not clear if endogenous T_3 contributed to the high D2 expression in TG myocardium. Furthermore, it might be expected that increased myocardial T_4 to T_3 conversion by hD2 might protect the heart against hypothyroidism. To explore these issues, three groups of TG and WT animals were kept on a low-iodine, PTU containing diet for 4, 8, and 12 days, respectively. This time was chosen based on preliminary experiments in which D2 activity fell to undetectable levels after 3 weeks on this regimen. D2 activity in the myocardium fell in parallel with the serum T_4 concentration with the lowest level found at 12 days (Fig. 4). These results confirmed the positive feedback of the D2 transgene by the endogenous thyroid hormone. At the same time, there was a time-dependent decrease in α-MHC and an increase in β-MHC mRNA levels in both TG and WT myocardium (Fig. 4). There was no statistically significant difference between WT and TG mice in the α-MHC, β-MHC, or SERCA mRNA level (by ANOVA, $P > 0.25$ for the effect of transgene on the expression of each mRNA) during induction of hypothyroidism suggesting that there was no protection of myocardial thyroidal status by myocardial hD2 (Fig. 4).

Discussion

D2 activity and mRNA level in the TG myocardium was extremely high, about 1000 times that in the normal euthyroid cortex with no activity detectable in WT hearts, confirming the strong transcriptional activity of α-MHC promoter. D2 activity was also present in lung because the

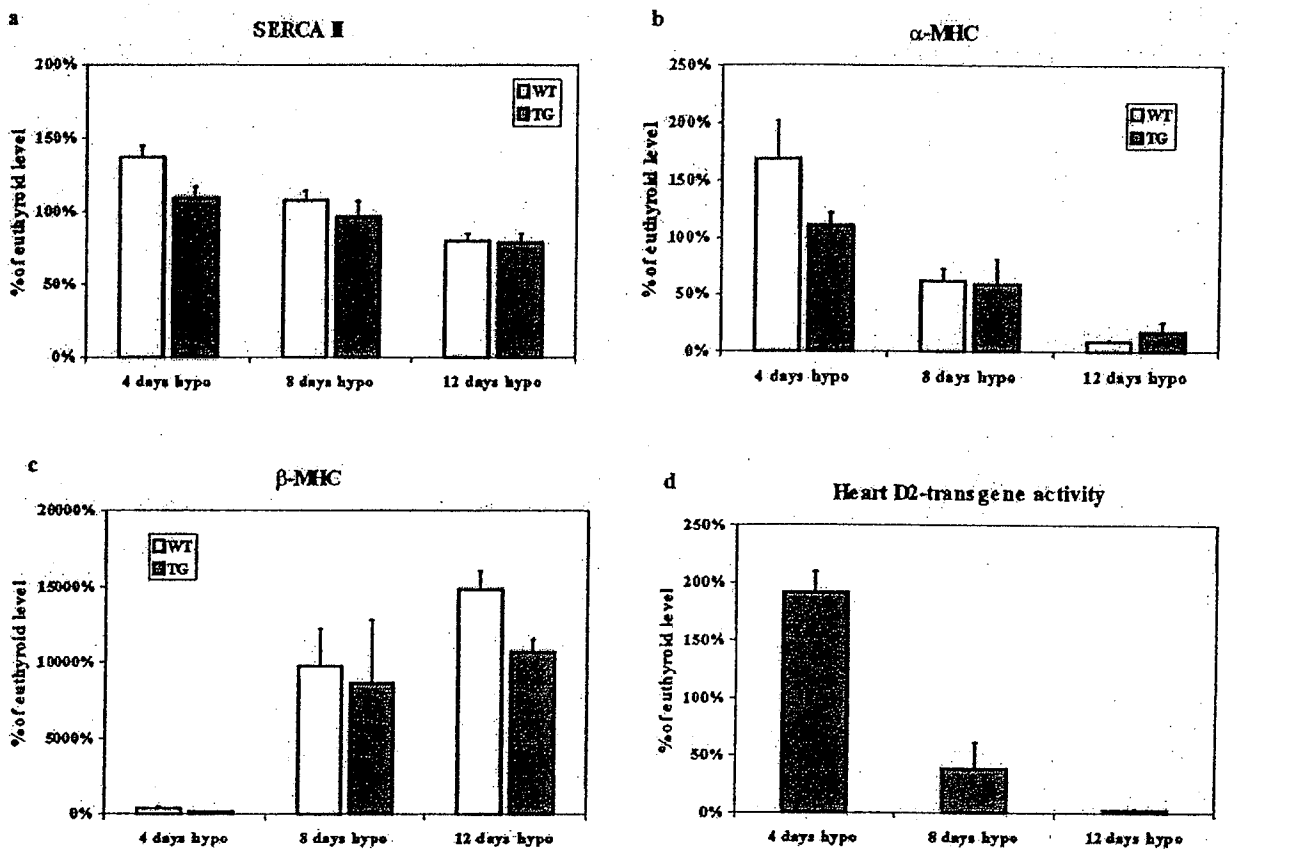


FIG. 4. a, Changes in SERCA II; b, α-MHC; and c, β-MHC mRNA during induction of hypothyroidism in WT and TG myocardium. All mRNA values are shown as a percentage of mean baseline mRNA level for TG mice. There were no significant differences between WT and TG mice as analyzed by ANOVA. d, Changes in D_2 transgene activity in TG hearts paralleled the changes in α-MHC mRNA.

α-MHC promoter also directs expression in the intimal walls of the veins and venules in that organ (29). The most surprising result of the experiment was the absence of a major effect on the myocardial T_3 concentration considering the level of D2 expression. In the cerebral cortex, for example, much lower D2 activity results in sufficient local T_3 production to saturate approximately 80% of the thyroid hormone receptors (9). The failure of tissue T_3 concentrations to rise to higher levels could have a number of explanations. D2 activity is expressed only in myocytes, which make up only about 50% of the myocardial cells and account for about 70% of the total myocardial proteins (3). Because the T_3 concentration was measured in whole heart homogenate and denominated by total myocardial protein, the difference between WT and TG is slightly underestimated. Nevertheless, even if corrected for the above factor, the increase in myocardial T_3 concentration is quite modest. This finding cannot be explained by the artifactual degradation of T_3 during tissue processing because there was no significant degradation of T_3 in tissue homogenate when incubated at 4 C for 6 h (data not shown). Nor can it be explained by intracellular T_4 to T_3 conversion in WT myocardium because this is not detectable.

There are other potential factors that could lead to lower T_3 production than one might anticipate based on the results of *in vitro* D2 assays. An as yet unidentified thiol-containing cellular cofactor is required for iodothyronine deiodination. Because the mouse myocyte does not normally convert T_4 to T_3, the level of this cofactor may be much lower than in brain, pituitary gland or brown fat. Alternatively, there may be limited T_4 uptake by the rodent myocyte. The molecular mechanism for T_4 and T_3 transport is only now being unraveled. One report indicates that T_4 transport into the heart is not temperature-dependent as is that of T_3, suggesting that it may not be an active process (31). Furthermore, when T_3 is produced, it may well diffuse rapidly from myocytes into the circulation due to the high myocardial blood flow. Although this is an attractive hypothesis, the similar concentrations of serum T_4 and T_3 in the sera of the TG and WT animals imply that the rate of total body conversion of T_4 to T_3 is not significantly increased by the expression of D2 in the myocardium. In all species examined to date, the fraction of T_4 converted to T_3 per 24 h in the whole animal is less than 50% (32, 33). If that fraction were to increase significantly, one would expect a downward adjustment of T_4 production by the hypothalamic-pituitary feedback system such that the serum T_4 might well be reduced with no change or perhaps a slight increase in serum T_3. The opposite change in serum T_4 has been documented in the C3H mouse in which a genetic decrease in D1 expression causes a 2-fold increase in circulating T_4 but no change in serum T_3 (34, 35). The intact mouse is a closed system so that thyroid status must remain constant

despite any changes in the relative fraction of T_4 to T_3 conversion.

Thyroid hormone response elements have been identified in the murine α-myosin heavy chain promoter, which was used to induce myocardial-specific D2 expression (29, 30). Thus, one would anticipate that a feed-forward mechanism might be present in the TG myocardium to increase D2 expression. The sensitivity of D2 expression to thyroid hormone is apparent from the results in Fig. 4. We took advantage of the sensitivity of the endogenous α- and β-MHC promoters to thyroid status to determine whether the expression of D2 would protect the myocardium against the effects of hypothyroidism. Although the thyroid hormone-dependence of D2 expression makes this experiment more problematic than it would be if D2 expression remained constant, we found no evidence that the transgene affects the onset of hypothyroidism-induced changes in myocardial gene expression (Fig. 4).

There was remarkably little effect of the transgene expression on the mouse. Growth rates were not different between TG and littermate controls and there was no alteration in the ratio of heart to body weight, which is a parameter commonly increased by excess thyroid hormone (36). This is consistent with the concept that the increased protein synthesis and hypertrophy of the hyperthyroid heart requires the increased myocardial work normally associated with systemic thyrotoxicosis (36). Unloading the heart by heterotopic cardiac transplantation has been shown to decrease overall protein synthesis and heart weight (37). In the same model, thyroid hormone excess induced increases in α-MHC mRNA is seen although unloading of the heart by itself leads to similar gene expression rearrangement as occurs in hypothyroidism (5). In euthyroid hearts, for example, there are minimal, if any, changes in α-MHC mRNA levels induced by treatment with excess thyroid hormone (7, 38).

The most striking evidence for myocardial thyrotoxicity of the TG animals was detected in the performance of the isolated heart. There was an approximately 20% increase in heart rate and an 30% increase in the rate pressure product (Fig. 2a). This result is consistent with earlier studies in isolated perfused rat hearts where alterations in thyroid status cause parallel alterations in basal heart rate (39, 40). It is also consistent with the current interpretation that the increase in heart rate induced by thyroid hormone is, at least partially, intrinsic to the muscle and does not require either changes in the autonomic nervous system or circulating catecholamines. A potential explanation for the increase in the intrinsic heart rate is the increased HCN2 expression found in TG animals. We have recently observed that HCN2 is thyroid hormone-responsive in rats although the major change in this mRNA, like that for α-MHC, occurs during the hypothyroid to euthyroid transition (7). In acutely thyrotoxic rats, there was a doubling of HCN2 mRNA from the hypothyroid to euthyroid state but only a 15% further increase during transition from euthyroidism to hyperthyroidism. Little is known of the factors regulating the mouse HCN2 gene. A recent communication suggests that it too may be thyroid hormone responsive. HCN2 mRNA levels were reduced about 50% in hypothyroid mice and were twice normal in hyperthyroid mice (41). In addition, the level of HCN2 mRNA was shown to be primarily regulated by α rather than by β thyroid hormone receptors (42). This could account for the fact that there was an increase in the mRNA for this gene but not that of α-MHC or SERCA II in the TG hearts. Such a species difference would also raise the possibility that in humans the HCN2 gene might also be positively regulated between the euthyroid and hyperthyroid state. This could account for the common observation of tachycardia as one of the earliest physical manifestations of thyrotoxicosis in humans. Although an increase in spontaneous heart rate correlated with the increase in HCN2 gene expression in the transgenic ventricles, it is well known that thyroid hormone action may be chamber specific and further studies analyzing HCN2 expression in atrial pacemaking cells will be needed to determine whether similar effects occur (43).

In association with the increased intrinsic heart rate, the ^{31}P NMR as well as biochemical measurements demonstrated a significant reduction in phosphocreatine in the transgenic hearts (Fig. 2, B and C, and Table 1). This was associated with a decrease in creatine level in the TG mice (Table 1). Both of those findings have been reported in the myocardium of hyperthyroid rats (20, 44). The decreased PCr may make the TG mouse heart more susceptible to ischemic challenge with a more rapid decrease in ATP and a greater increase in Pi than occurs under normal circumstances. Such effects could then lead to decreases in myocardial pH and reduced cardiac function (25). Testing to determine the validity of such predictions is currently in progress.

The changes, such in myocardial performance and biochemistry, induced by chronic D2 overexpression are unexpected. Some of the more striking alterations in gene expression expected on the basis of earlier short-term, high dose of exogenous thyroid hormones did not occur. In humans, only modest increases in serum T_3 and T_4 (within the normal range) are required to cause suppression of TSH. There is considerable controversy about whether or not such subclinical hyperthyroidism, manifested only by a suppressed TSH, is physiologically significant (45). Because the changes demonstrated in these mice occur with minimal increases in myocardial T_3 together with the fact that the human myocardium also expresses D2 mRNA, modest increases in circulating T_4 and T_3 would have similar effects on the human myocardium. Supporting this is a recent report that in a group of patients with normal thyroid hormone levels but suppressed TSH, 24-h Holter monitoring showed an increase in heart rate from 71 to 82 beats per minute compared with age-matched controls (45). Thus, mice expressing a D2 transgene may provide a model for evaluation of the consequences of mild chronic thyrotoxicosis on myocardial function which is hard to generate by any other technique.

References

1. Larsen PR, Davies TF, Hay ID 1998 The thyroid gland. In: Wilson JD, Foster DW, Kronenberg HM, Larsen PR (eds) Williams Textbook of Endocrinology, ed 9. WB Saunders Co., Philadelphia, pp 389–515
2. Klein I 1990 Thyroid hormone and the cardiovascular system. Am J Med 88:631–637
3. Dillmann WH 1990 Biochemical basis of thyroid hormone action in the heart. Am J Med 88:626–630
4. Rohrer DK, Hartong R, Dillmann WH 1991 Influence of thyroid hormone and retinoic acid on slow sarcoplasmic reticulum Ca^{2+} ATPase and myosin heavy chain alpha gene expression in cardiac myocytes. Delineation of cis-active

DNA elements that confer responsiveness to thyroid hormone but not to retinoic acid. J Biol Chem 266:8638–8646
5. Ojamaa K, Klein I 1993 In vivo regulation of recombinant cardiac myosin heavy chain gene expression by thyroid hormone. Endocrinology 132: 1002–1006
6. Tsika RW, Bahl JJ, Leinwand LA, Morkin E 1990 Thyroid hormone regulates expression of a transfected human alpha-myosin heavy-chain fusion gene in fetal rat heart cells. Proc Natl Acad Sci USA 87:379–383
7. Pachucki J, Burmeister LA, Larsen PR 1999 Thyroid hormone regulates hyperpolarization-activated cyclic nucleotide-gated channel (HCN2) mRNA in the rat heart. Circ Res 85:498–503
8. Larsen PR, Berry MJ 1994 Type I iodothyronine deiodinase: unexpected complexities in a simple deiodination reaction. Thyroid 4:357–362
9. Larsen PR, Silva JE, Kaplan MM 1981 Relationships between circulating and intracellular thyroid hormones: physiological and clinical implications. Endocr Rev 2:87–102
10. Riesco G, Taurog A, Larsen PR, Krulich L 1977 Acute and chronic responses to iodine deficiency in rats. Endocrinology 100:303–313
11. Buettner C, Harney JW, Larsen PR 1998 The 3′-untranslated region of human type 2 iodothyronine deiodinase mRNA contains a functional selenocysteine insertion sequence element. J Biol Chem 273:33374–33378
12. Salvatore D, Bartha T, Harney JW, Larsen PR 1996 Molecular biological and biochemical characterization of the human type 2 selenodeiodinase. Endocrinology 137:3308–3315
13. Croteau W, Davey JC, Galton VA, St Germain DL 1996 Cloning of the mammalian type II iodothyronine deiodinase. A selenoprotein differentially expressed and regulated in human and rat brain and other tissues [see comments]. J Clin Invest 98:405–417
14. Salvatore D, Tu H, Harney JW, Larsen PR 1996 Type 2 iodothyronine deiodinase is highly expressed in human thyroid. J Clin Invest 98:962–968
15. Davey JC, Schneider MJ, Becker KB, Galton VA 1999 Cloning of a 5.8 kb cDNA for a mouse type 2 deiodinase. Endocrinology 140:1022–1025
16. Abrams GM, Larsen PR 1973 Triiodothyronine and thyroxine in the serum and thyroid glands of iodine-deficient rats. J Clin Invest 52:2522–2531
17. Larsen PR 1971 Inhibition of triiodothyronine (T_3) binding to thyroxine-binding globulin by sodium salicylate and its application to immunoassay of T_3 in human serum. Metabolism 20:976–980
18. Larsen PR 1972 Direct immunoassay of triiodothyronine in human serum. J Clin Invest 51:1939–1949
19. Spindler M, Saupe KW, Christe ME, Sweeney HL, Seidman CE, Seidman JG, Ingwall JS 1998 Diastolic dysfunction and altered energetics in the αMHC403/+ mouse model of familial hypertrophic cardiomyopathy. J Clin Invest 101:1775–1783
20. Bak MI, Ingwall JS 1998 Regulation of cardiac AMP-specific 5′-nucleotidase during ischemia mediates ATP resynthesis on reflow. Am J Physiol 274:C992–C1001
21. Saupe KW, Spindler M, Tian R, Ingwall JS 1998 Impaired cardiac energetics in mice lacking muscle-specific isoenzymes of creatine kinase. Circ Res 82:898–907
22. Veech RL, Lawson JW, Cornell NW, Krebs HA 1979 Cytosolic phosphorylation potential. J Biol Chem 254:6538–6547
23. Kammermeier H, Schmidt P, Jungling E 1982 Free energy change of ATP-hydrolysis: a causal factor of early hypoxic failure of the myocardium? J Mol Cell Cardiol 14:267–277
24. Passonneau JV, Lowry OH 1993 Enzymatic Analysis: A Practical Guide. Humana Press, Totowa, NJ
25. Ingwall JS, Kramer MF, Fifer MA, Lorell BH, Shemin R, Grossman W, Allen PD 1985 The creatine kinase system in normal and diseased human myocardium. N Engl J Med 313:1050–1054
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the folin-phenol reagents. J Biol Chem 193:265–275
27. Silva JE, Leonard JL, Crantz FR, Larsen PR 1982 Evidence for two tissue specific pathways for *in vivo* thyroxine 5′deiodination in the rat. J Clin Invest 69:1176–1184
28. Robbins J, Gulick J, Sanchez A, Howles P, Doetschman T 1990 Mouse embryonic stem cells express the cardiac myosin heavy chain genes during development in vitro. J Biol Chem 265:11905–11909
29. Subramaniam A, Jones WK, Gulick J, Wert S, Neumann J, Robbins J 1991 Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. J Biol Chem 266:24613–24620
30. Izumo S, Nadal-Ginard B, Mahdavi V 1986 All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. Science 231:597–600
31. Everts ME, Verhoeven FA, Bezstarosti K, Moerings EP, Hennemann G, Visser TJ, Lamers JM 1996 Uptake of thyroid hormones in neonatal rat cardiac myocytes. Endocrinology 137:4235–4242
32. Inada M, Kasagi K, Kurata S, Kazama Y, Takayama H, Torizuka K, Fukase M, Soma T 1975 Estimation of thyroxine and triiodothyronine distribution and of the conversion rate of thyroxine to triiodothyronine in man. J Clin Invest 55:1337–1348
33. Oppenheimer JH, Schwartz HL, Surks MI 1972 Propylthiouracil inhibits the conversion of L-thyroxine to L-triiodothyronine: an explanation of the antithyroxine effect of propylthiouracil and evidence supporting the concept that triiodothyronine is the active thyroid hormone. J Clin Invest 51:2493–2497
34. Berry MJ, Grieco D, Taylor BA, Maia AL, Kieffer JD, Beamer W, Glover E, Poland A, Larsen PR 1993 Physiological and genetic analyses of inbred mouse strains with a type I iodothyronine 5′ deiodinase deficiency. J Clin Invest 92:1517–1528
35. Maia AL, Kieffer JD, Harney JW, Larsen PR 1995 Effect of 3,5,3′-triiodothyronine (T_3) administration on dio1 gene expression and T_3 metabolism in normal and type 1 deiodinase-deficient mice. Endocrinology 136:4842–4849
36. Klein I 1988 Thyroxine-induced cardiac hypertrophy: time course of development and inhibition by propranolol. Endocrinology 123:203–210
37. Ojamaa K, Samarel AM, Kupfer JM, Hong C, Klein I 1992 Thyroid hormone effects on cardiac gene expression independent of cardiac growth and protein synthesis. Am J Physiol 263:E534–E540
38. Ojamaa K, Klein I 1991 Thyroid hormone regulation of alpha-myosin heavy chain promoter activity assessed by *in vivo* DNA transfer in rat heart. Biochem Biophys Res Commun 179:1269–1275
39. Read LC, Wallace PG, Berry MN 1987 Effects of thyroid state on respiration of perfused rat and guinea pig hearts. Am J Physiol 253:H519–H523
40. McDonough KH, Chen V, Spitzer JJ 1987 Effect of altered thyroid status on in vitro cardiac performance in rats. Am J Physiol 252:H788–H795
41. Gloss B, Sayen MR, Trost SU, Bluhm WF, Meyer M, Swanson EA, Usala SJ, Dillmann WH 1999 Altered cardiac phenotype in transgenic mice carrying the δ337 threonine thyroid hormone receptor beta mutant derived from the S family. Endocrinology 140:897–902
42. Gloss BR, Swanson EA, Trost SU, Chassande O, Samarut J, G. C, Scanlan TS, Baxter JD, Dillmann WH, Absent response of the cardiac pacemaker channel HCN2 and heart rate to a thyroid hormone receptor β preferred ligand GC-1. Program of the 82nd Annual Meeting of The Endocrine Society, Toronto, 2000, p 2370
43. Ojamaa K, Sabet A, Kenessey A, Shenoy R, Klein I 1999 Regulation of rat cardiac Kv1.5 gene expression by thyroid hormone is rapid and chamber specific. Endocrinology 140:3170–3176
44. Lortet S, Heckmann M, Ray A, Rossi A, Aussedat J, Grably S, Zimmer HG 1995 Energy metabolism response to calcium activation in isolated rat hearts during development and regression of T_3-induced hypertrophy. Mol Cell Biochem 151:99–106
45. Biondi B, Fazio S, Palmieri EA, Tremalaterra R, Angellotti G, Bone F, Riccio G, Cittadini A, Lombardi G, Sacca L 1999 Effects of chronic subclinical hyperthyroidism from levothyroxine on cardiac morphology and function. Cardiologia 44:443–449

EXHIBIT F

Catheter-Delivered *In Vivo* Gene Transfer into Rat Myocardium Using the Fusigenic Liposomal Mediated Method

Ken SHINMURA, MD, Ryuichi MORISHITA, MD, Motokuni AOKI, MD, Jitsuo HIGAKI, MD, Toshio OGIHARA, MD, Yasufumi KANEDA,[1] MD, *and* Masato TANI, MD

SUMMARY

We compared the efficacy of four different *in vivo* hemagglutinating virus of Japan (HVJ)-liposome gene transfer methods, i.e., direct myocardial injection (IM), injection into the left ventricular cavity (LV), infusion at the level of the coronary cusps (CI), or injection into the left ventricular cavity with a balloon catheter blocking aortic flow (LV+B) to transfer β - galactosidase, FITC-labeled oligodeoxynucleotide (ODN), and / or luciferase genes into the rat heart. IM caused highly efficient gene transfer in the limited area around the injection site, which suggests that IM may be a suitable method for targeted treatment of focal lesion. In the LV+B group, all rats had myocardial β - galactosidase staining and fluorescence of FITC-labeled ODN in the nuclei of cardiac myocytes around the coronary arteries and the vasa vasorum, and some transfected myocytes were observed in the middle of the myocardium without any evidence of injury. In contrast, in the CI group, only half of the animals had myocardial expression of β - galactosidase. In contrast, fluorescence or luciferase activity was present throughout the left ventricle in the LV+B group. However, the percentage of myocytes that exhibited fluorescence was less than 1% of the total ventricular myocyte population and luciferase activity in the LV+B group was 1.6% of that in the IM group. No evidence of luciferase expression was observed in brain, lung, liver, kidney, or testis in either the IM or LV+B group. These results suggest that HVJ-liposome gene transfer into the myocardium through the coronary arteries using a balloon-catheter technique is safe and has the potential for causing widespread transgene expression with organ-specificity, although the efficiency of gene transfer should be improved. (Jpn Heart J 2000; 41: 633-647)

Key words: Gene therapy, Gene expression, Histopathology, Myocytes, Coronary circulation

RECENT advances in molecular biology techniques have provided genetic information about cardiovascular diseases. Further, gene therapy represents a

From the, Department of Geriatric Medicine, Keio University School of Medicine, Tokyo, [1] Institute for Molecular and Cellular Biology, Osaka University Medical School, Osaka, Japan.

Address for correspondence: Masato Tani, MD, Department of Geriatrics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.

Received for publication May 17, 2000.

Revised and accepted June 1, 2000.

potential strategy for the treatment of cardiovascular diseases.[1,2] In fact, several gene transfer methods, including adenoviral and liposomal transfer, are being used clinically for human gene therapy for certain diseases. However, the use of gene therapy to treat cardiac diseases has been limited because it is very difficult to transfect cardiac myocytes efficiently *in vivo*. Recent reports have suggested that adenoviral vectors can be used as an efficient means of myocardial transfection via direct injection or coronary infusion.[3-7] However, because of theoretical disadvantages, in addition to concerns about safety,[8,9] there are continued efforts to develop novel vector systems for myocardial transfection.

We have previously demonstrated efficient gene transfer into the rat heart using a Hemagglutinating Virus of Japan (HVJ)-liposome method that has been used to transfect blood vessels, liver, and kidney. We have employed three different approaches to transfect the heart using HVJ-liposomes: direct myocardial injection, coronary infusion, and pericardial incubation.[10] However, we have not compared the transfection efficiency of the three approaches. In addition, it is important to develop catheter-based methods for the clinical applications of gene transfer since direct myocardial injection or pericardial incubation may potentially damage coronary arteries or provoke arrhythmias while catheter-based methods can be used during a usual catheterization procedure. Development of *in vivo* methods of gene transfer via catheter-based techniques may provide an approach for treating myocardial diseases including myocardial infarction and cardiomyopathy.

In this study we evaluated the transfection efficiency of four different transfer methods: 1) direct injection into the myocardium, 2) injection into the left ventricular cavity, 3) infusion at the level of the coronary cusps, and 4) injection into the left ventricular cavity with blockade of aortic flow by a balloon catheter. We assessed transfection efficiency using the luciferase gene, as well as the tissue distribution of transgene expression and oligodeoxynucleotide (ODN) distribution using the β - galactosidase gene and FITC-labeled ODN, respectively. Moreover, the tissue-specificity of transgene expression using these approaches was also examined.

MATERIALS AND METHODS

Construction of plasmids and FITC-labeled ODN: We obtained a β - galactosidase gene expression vector driven by the SV 40 promoter (LacZ Ncl) from a commercial source (Promega Corporation, Madison, WI). The luciferase gene expression vector was driven by the EB virus promoter (pEBT LuCNII).[10] We also constructed a control expression vector without

the luciferase gene driven by the EB virus promoter. FITC-labeled ODN were purchased from Nihon-Seifun (Gunma, Japan). FITC was added to the 3' and 5' ends of the ODN (16 base pairs: 5'-CCT-TGA-AGG-GAT-TTC-CCT-CC-3'). The ODN were lyophilized, resuspended in PBS, and quantified by spectrophotometry.[11-13]

Preparation of HVJ-liposome: HVJ (Sendai virus; Z strain) was propagated in chorioallantoic fluid of embryonated eggs.[11-13] Briefly, HVJ was collected by centrifugation at 27,000 g for 40 minutes and resuspended overnight in BSS(–) (137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6). This procedure was repeated at least twice. The resuspended HVJ was stored at –4°C and used within 1 week after purification. The hemagglutinating activity of HVJ was determined as described previously.[11-14] A measurement of 1 absorbance unit at 540 nm for an HVJ suspension (1 mg / m*l* protein) was equivalent to 15,000 HAU / m*l* of fusigenic activity. The preparation of the HVJ-liposome complex has been previously described.[11-20] Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in tetrahydrofuran at a weight ratio of 1 : 4.8 : 2. The lipid mixture (10 mg) was deposited on the sides of a flask by removal of the tetrahydrofuran using a rotary evaporator. The dried lipid was hydrated in 200 μl of balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 13 mM Tris-HCl, pH 7.6) containing plasmid-HMG (high mobility group)-1 complex (200 μg : 64 μg), which had previously been incubated at 20°C for 1 hr. Liposomes were prepared by shaking and sonicating the mixture. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg / mm^2 / sec) for 3 min just before use. The liposome suspension (0.5 m*l*, containing 10 mg of lipid) was mixed with HVJ (30,000 HAU) in a total volume of 2 m*l* of BSS. The mixture was incubated at 4°C for 10 min and then for 30 min with gentle shaking at 37°C. The Free HVJ was separated from the HVJ-liposome complex by sucrose density gradient centrifugation. The final concentrations used in the present study were 20 μg / m*l* for the HVJ-liposome complex and 1 μM for the FITC-ODN. We used HMG-1 with HVJ-liposome complex to enhance the migration of plasmid DNA into the nucleus.[15,21] However, HMG-1 was not used for ODN transfer because ODN can easily migrate into the nucleus without HMG-1.

***In vivo* myocardial gene transfer:** Male Sprague-Dawley rats (300 to 350 g; Charles River Breeding Laboratories, Kanagawa, Japan) were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg /kg). Rats were then intubated and mechanically ventilated. Following the transfection summarized below and in Figure 1, rats were sacrificed 5 days (plas-

mids) or 2 days (FITC-labeled ODN) after transfection. The protocols used in the present study conform to the principles set forth in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Direct myocardial injection (IM group; n=15): HVJ-liposomes (400 μl) were carefully injected through a left lateral thoracotomy directly into the anterior wall and apex of the heart using a 30G needle. The chest incision was then closed.

Direct injection into the left ventricular cavity (LV group; n=12): HVJ - liposomes (800 μl) were carefully injected directly into the left ventricular cavity through the anterior wall using a 30G needle via a left lateral thoracotomy. The chest incision was then closed.

Aortic cusp infusion (CI Group; n=10): The right common carotid artery was surgically exposed. A cannula (PE50) was introduced into the common carotid artery, and advanced to the level of the aortic valve through the ascending aorta. The cannula was positioned at the aortic cusp just above the aortic valve for infusion of the HVJ-liposome complex (800 μl) over 10 sec at room temperature. After infusion, the cannula was removed. No adverse neurologic or vascular effects were observed in any of the animals undergoing this procedure.

Direct injection into the left ventricular cavity with blockade of ascending aortic flow by a balloon catheter (LV+B Group; n=13): The right common carotid artery was surgically exposed. A balloon catheter (Fogarty 2F, Baxter, Tokyo, Japan) was introduced into the common carotid artery and advanced to the level of the aortic valve as described above. The balloon was inflated with 0.3 m*l* of air and positioned at takeoff of the brachiocehalic artery during the infusion of the HVJ-liposome complex. HVJ-liposomes (800 μl) were injected directly into the left ventricular cavity as described above. The chest incision was then closed and the balloon catheter removed.

Analysis of luciferase activity: Firefly luciferase activity was measured using a luciferase assay system (PicaGeneTM; Toyo-Inki, Tokyo, Japan).[10] Rats were sacrificed 5 days after transfection as described above. The hearts were rapidly excised and frozen in liquid nitrogen. In some rats, the entire left ventricle was homogenized in lysis buffer. In other rats, the left ventricle was divided into four regions: anterior wall, septum, posterior wall, and apex. Each portion was homogenized separately in lysis buffer. The brain, lung, liver, kidney, and testis from some of the rats in the IM and LV+B groups were isolated and homogenized in lysis buffer. Tissue lysates were centrifuged (3000 rpm, 10 min), and 20 μl of super-

natant was mixed with 100 μl of luciferase assay reagent. The measurement of luminescence was initiated 5 sec after addition of sample and continued for 10 sec. The chemiluminescence (light intensity) produced during 10 sec was used for the calculation of luciferase activity. The luciferase activity in each sample was corrected for protein content and expressed as light intensity / mg protein.

Staining for β - Galactosidase: Histochemical analysis for β - galactosidase was performed 7 days after transfection with the HVJ-liposome complex containing the β - galactosidase gene.[10] Rats were sacrificed 3 days after transfection and tissues were fixed with 1% glutaraldehyde in PBS for 3 hr at 4°C. After fixation, the tissues were processed in a standard manner. Specimens were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside as previously described.[20] Sufficient buffered X-gal chromagen solution was added to cover the tissue slices. Buffered X-gal solution was prepared from solution A and solution B (A : B = 1 : 99) [solution A : 49 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside dissolved in 1 ml dimethylformamide, solution B : 1 M $MgCl_2$ and 3 M $K_4Fe(CN)_6$ dissolved in PBS]. After an overnight incubation, the sections were mounted and analyzed by microscopy. In order to determine the degree of background staining, hearts transfected with HVJ-liposome complex containing a control vector lacking the β - galactosidase gene and untransfected hearts were processed in the same manner.

Morphologic analysis of FITC-ODN: HVJ-liposome complex containing FITC-labeled ODN (1 μM) was transfected using one of two methods: 1) direct myocardial injection (IM group; Figure 1A), and 2) direct injection into the left ventricular cavity with blockade of ascending aortic flow by a balloon catheter (LV+B Group; Figure 1D). Rats were sacrificed, perfusion-fixed with 4% paraformaldehyde, and the tissue processed in a standard manner. Sections were examined by fluorescence microscopy after staining with eriochrome black T solution. Elastic fibers, which stain dark red with eriochrome black T solution, were readily distinguishable from the myocytes containing FITC-labeled ODN.[12,13]

Statistical analysis: All values are expressed as the mean ± SEM. Analysis of variance with subsequent Bonferroni's test was used for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

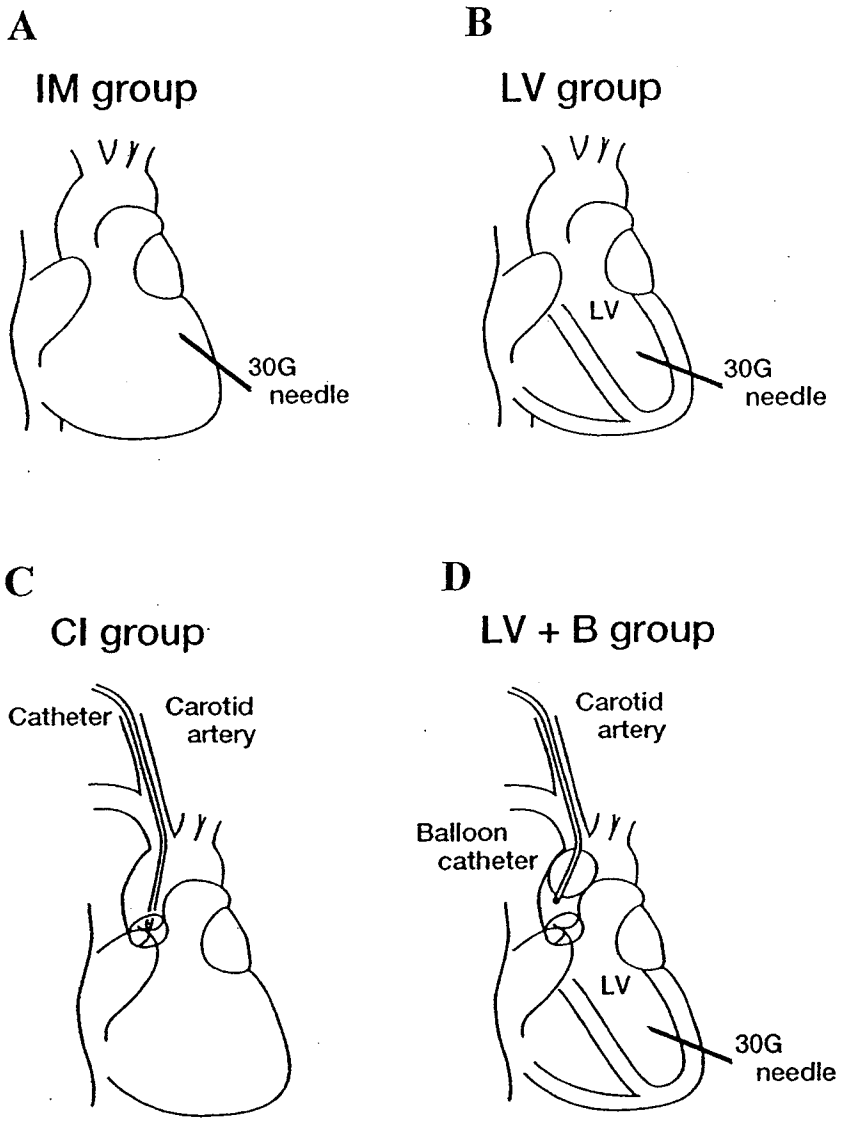


Figure 1. Schema for in vivo gene transfer. **A:** IM group: direct myocardial injection. **B:** LV group: Injection into the left ventricular cavity. **C:** CI group: the aortic cuspid infusion. **D:** LV+B group: Injection into the left ventricular cavity with blocking of ascending aortic flow by a balloon catheter.

RESULTS

β-Galactosidase staining in the myocardium: Direct injection of the HVJ-liposome complex containing the β-galactosidase vector into the myocardium (IM group) resulted in intense β-galactosidase staining at the injec-

Figure 2. Staining for β - galactosidase activity in hearts transfected with the β - galactosidase gene in the IM and LV+B groups. (A) β - galactosidase staining of myocytes transfected with control vector (× 200) in the IM group; (B) β - galactosidase staining in the IM group (× 200, arrow indicates the injection site); (C) β - galactosidase staining in the LV+B group (× 200, arrow indicates positively stained myocytes around the coronary arteries and the vasa vasorum).

tion site 5 days after transfection (Figure 2B), which is consistent with our previous findings.[10] All of the rats in the IM group exhibited evidence of myocardial staining. Although the percentage of cells around the injection site that contained β - galactosidase was very high (approximately 80%), the staining was limited to around the injection site. In addition, significant injury and fibrosis were observed at the injection site (arrow in Figure 2B shows the injection site). In order to avoid injury and fibrosis by direct injection, we also performed direct injection into the left ventricular cavity (LV group). Compared to the IM group, there were fewer myocytes that contained β - galactosidase. Although the staining was detected primarily in myocytes around the coronary arteries (data not shown), β - galactosidase activity was present in only 1 of the 4 rats in the LV group (25%).

Based on these results, we attempted to infuse the HVJ-liposome complex into the coronary arteries for *in vivo* myocardial gene transfer. Direct infusion of HVJ-liposome complex at the level of the aortic cusp (CI group) resulted in staining of myocytes for β - galactosidase activity, but with no evidence of injury (data not shown). Myocytes staining for β - galactosidase were observed around the coronary arteries and vasa vasorum. However, only 2 of the 4 rats (50%) transfected with the β - galactosidase vector exhibited evidence of staining. The low frequency of transfection may be due to technical difficulties associated with maintaining the position of the catheter in the coronary artery.

To increase the volume of HVJ-liposome complex delivered into the coronary arteries, we injected the HVJ-liposome complex into the left ventricular cavity while blocking ascending aortic flow with a balloon catheter (LV+B group). Numerous cardiac myocytes stained for β - galactosidase activity around the coronary arteries and vasa vasorum (Figure 2C, arrow). Furthermore, transfected myocytes were observed in the middle of the myocardium (Figure 2C). All of the rats transfected with the β - galactosidase vector in the LV+B group exhibited evidence of staining (100%). There were no myocytes that stained for β - galactosidase activity in rats transfected with a control vector (Figure 2A) or in untransfected rats (data not shown). No evidence of cytotoxicity or inflammation caused by the HVJ-liposome complex was observed in the LV+B, IC, or LV groups.

Comparison of FITC-labeled ODN distribution between the IM and LV+B groups: To characterize the localization of transfected cells further, FITC-labeled ODN were also used to transfect cells. As shown in Figure 3C, fluorescence was observed in the nuclei of cardiac myocytes in both the IM and LV+B groups 2 days after transfection. In the IM group, myocytes show-

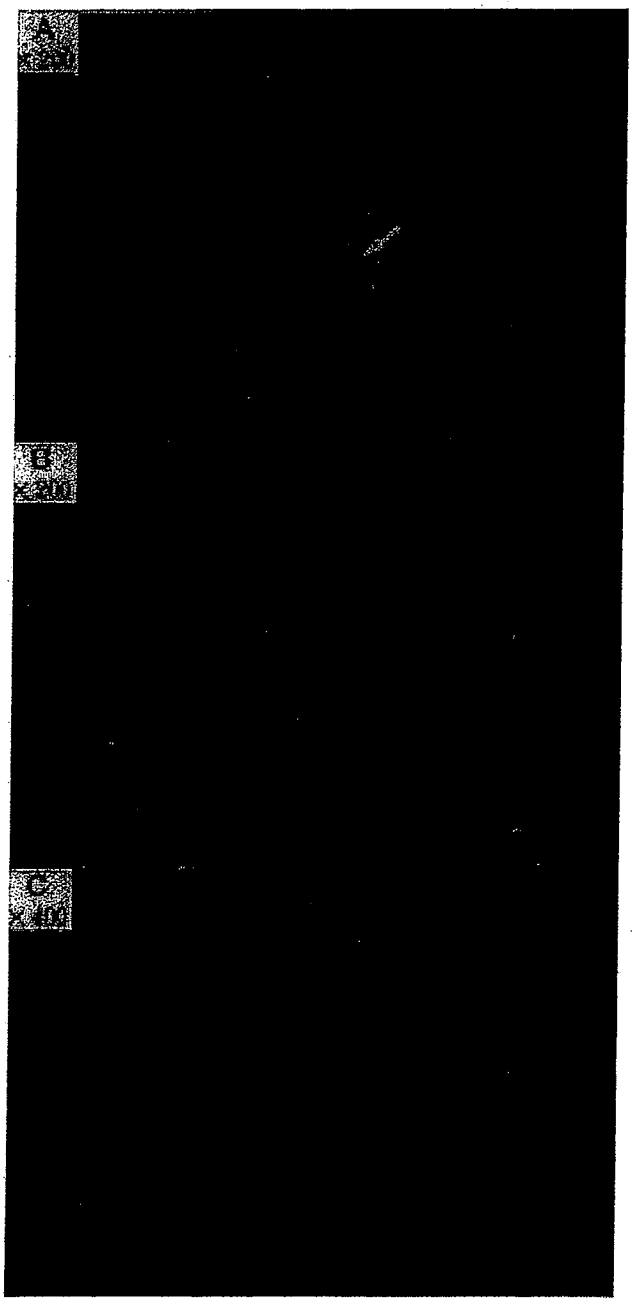


Figure 3. Fluorescence in hearts transfected with FITC-labeled ODN in the IM and LV+B groups. **A:** Fluorescence at the injection site in the IM group (× 200, arrow indicates the injection site). **B:** Myocardial fluorescence in the LV+B group (× 200). **C:** Myocardial fluorescence in the LV + B group (× 400, fluorescence was observed in the nuclei of cardiac myocytes and endothelial cells).

ing fluorescence were limited to the area around the injection site. However, fluorescence was observed in the nuclei of approximately 90% of the myocytes around the injection site (Figure 3B). In contrast, myocyte fluorescence was present throughout the left ventricle in the LV+B group. The percentage of myocytes that exhibited fluorescence was < 1% of the total ventricular myocyte population. In addition to myocytes, endothelial cells in the capillaries also exhibited round fluorescence in the LV+B group (Figure 3C). In contrast, few of the endothelial cells in the IM group exhibited fluorescence.

Transfection efficiency assessed by luciferase activity: We compared the transfection efficiency of the 4 methods by determining luciferase activity in the whole left ventricle (Table). In the LV+B group, luciferase activity was 1.6% of the value for the IM group ($p < 0.01$), but was still significantly greater than in the LV or CI groups (data not shown). When comparing the distribution of luciferase activity in the different regions of the left ventricle, luciferase activity was detected only in the anterior wall and apex around the injection site in the IM group (Figure 4A). In the LV+B group (Figure 4B), luciferase activity was detected in all 4 regions. Luciferase activity was greatest in the apex and lowest in the posterior wall in the LV+B groups, however, the difference did not reach statistical significance. In contrast to the heart, luciferase activity was not detected in other tissues (brain, lung, liver, kidney and testis) in either the IM or LV+B group.

Table. Comparison of Luciferase Activities Among the 4 Transfected Groups

	IM group	LV group	LV+B group	CI group
Number	6	6	4	4
HVJ-liposome Solution applied (μl)	400	800	800	800
Light intensity / mg protein	24220 ± 5970	30 ± 30*#	770 ± 260*	90 ± 110*#
Light intensity / mg protein / ml HVJ-liposome solution	60550 ± 13500	40 ± 30*#	960 ± 320*#	110 ± 140*#
Relative luciferase activity (%)	100	0.1	1.6	0.2

Groups are as defined in Figure 1. * : $p < 0.05$ vs IM group, # : $p < 0.05$ vs LV+B group.

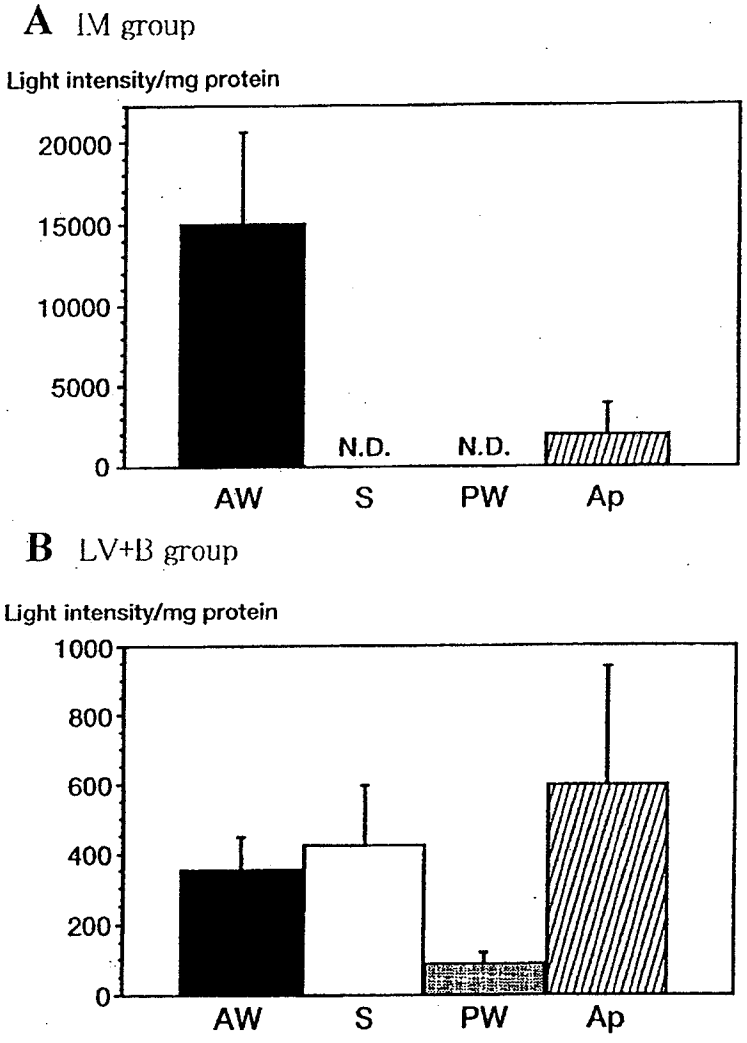


Figure 4. Distribution of luciferase activity in different regions of the left ventricle in the IM (A) and the LV+B (B) groups. AW=anterior wall; S=septum; PW=posterior wall; Ap=apex; ND=not detected.

DISCUSSION

The ability to express recombinant genes in the coronary vascular system and the myocardium holds promise for the treatment of a number of acquired and inherited cardiovascular diseases. Previous *in vivo* gene transfer approaches used in the heart have been limited by a relatively low efficiency of gene transduction.[8,9] Recently, many researchers have

focused on the use of adenoviral vectors, because they can effectively transfect in the myocardium.[3-7] However, use of these vectors has some theoretical disadvantages including 1) induction of inflammation and 2) production of neutralizing antibodies.[8,9] Therefore, it is important to develop an efficient and safe gene transfer method for the heart.

The HVJ-liposome method has been shown to be an efficient gene transfer method in blood vessels, the kidney, and the liver.[11-18,21] The method utilizes a cell-membrane fusion mechanism which bypasses endocytotic uptake.[12,13,22] This approach takes advantage of the ability of the HVJ (also known as Sendai virus) envelope to fuse with the cell membrane at a neutral pH.[12,13,22] Moreover, repeated injections of HVJ-liposome solution do not attenuate transgene expression, suggesting that neutralizing antibodies are not produced.[23] Recently, we reported that the HVJ-liposome method is an efficient and less toxic *in vivo* myocardial gene transfer method.[10] We have previously employed three different methods of transfection using direct injection, coronary infusion and pericardial incubation.[10] However, we did not compare the transfection efficiencies of these approaches. Moreover, it is important to develop catheter-based approaches for myocardial gene transfer for use in clinical human gene therapy. In addition, it is important to develop catheter-based methods for the clinical applications of gene transfer since direct myocardial injection or pericardial incubation may damage coronary arteries or provoke arrhythmias during open chest surgery while catheter-based methods can be used during usual catheterization procedures. Therefore, in the present study, we evaluated the transfection efficiency of four different approaches for *in vivo* gene transfer in the rat myocardium: 1) direct injection into the myocardium (IM group), 2) injection into the left ventricular cavity (LV group), 3) infusion at the level of the coronary cusps (CI group), and 4) injection into the left ventricular cavity with blocking of aortic flow by a balloon catheter (LV+B group).

The present study demonstrated that direct myocardial injection was the most effective method for increasing transgene expression in a limited area (Table). The high transfection efficiency of intramyocardial injection that we found in the present study is consistent with the findings of previous reports.[24-31] However, significant myocardial damage, as evidenced by the accumulation of neutrophils and necrosis, was observed at the injection site,[24-29] although recent reports may suggest that the focal damage at the injection site may be negligible.[30,31] Another problem in the IM group was that the area of transfection was very limited around the injection site in the present study (Figure 2), also in keeping with previous

reports,[24-31] which suggests that IM may be the best method for targeted treatment of focal lesions. There were marked differences in the β - galactosidase activity in the transfected areas between the different approaches (Figures 2 & 3). In the LV+B group, staining for β - galactosidase was observed in cardiac myocytes both near microvessels as well as far away from capillaries. This finding would suggest that the HVJ-liposome complex can penetrate the endothelium of microvessels in the heart, and transfect myocytes. The ability of HVJ-liposomes to penetrate endothelial cells is consistent with previous reports of *in vivo* gene transfer in blood vessels.[11,13,16,19] In contrast, recent reports of *in vivo* gene transfer in uninjured vessels using adenoviral vectors demonstrated that the transgene localizes to the endothelial layer.[32,33] Therefore, adenoviral vectors do not penetrate but are trapped in the endothelial cells. This difference in distribution may be due to differences in the characteristics of the vector systems (adenovirus vs HVJ). More importantly, endothelial cells in capillaries could be efficiently transfected by injection into the left ventricular cavity with blocking of aortic flow by a balloon catheter.

Infusion of transgenes into the coronary arteries results in the widespread expression of transgenes in cardiac myocytes as compared with direct myocardial injection.[10,34] However, comparison of the transfection efficiencies of direct myocardial injection and infusion into the coronary arteries has not previously been examined. The present study demonstrated low transfection efficiencies in the LV and CI groups, which may be explained by incomplete injection of the HVJ-liposome complex into the coronary arteries. This hypothesis is supported by the observation that the CI group had a lower transfection efficiency than the LV+B group. Therefore, if a smaller double-lumen balloon catheter is developed, injection into the coronary arteries with blocking of aortic flow by the balloon may be the optimum method to transfect the myocardium and endothelial cells. Previous reports have demonstrated a high transfection efficiency with coronary artery infusion in a transplant model.[35] An added benefit of this method is the lack of cytotoxicity and inflammation with the use of HVJ-liposome complex.

Following the intracoronary infusion of adenovirus, wide-spread transgene expression can be detected in the liver, kidneys, lungs, brain and testis of animals 5 days after virus infusion.[6] In contrast, transfection of the luciferase gene using the HVJ-liposome method resulted in no luciferase activity in any organ except the heart. This myocardium-specific transfection would be useful in the study of the effects of gene products in the myocardium in the absence of systemic gene expression. Percuta-

neous transluminal gene transfer into the heart by intracoronary infusion of HVJ-liposome vectors may represent an efficient method of introducing recombinant genes into the coronary arterial wall and the surrounding myocardium.

ACKNOWLEDGEMENT

This work was supported in part by grants from the Keio Health Consulting Center and the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1. Anderson WF. Human gene therapy. Science 1992; 256: 808-13.
2. Miller AD. Human gene therapy comes of age. Nature 1992; 357: 455-60.
3. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circ Res 1993; 73: 1202-7.
4. Kirshenbaum LA, MacLellan WR, Mazur W, French BA, Schneider MD. Highly efficient gene transfer into adult ventricular myocytes by recombinant adenovirus. J Clin Invest 1993; 92: 381-7.
5. Schneider MD, French BA. The advent of adenovirus gene therapy for cardiovascular disease. Circulation 1993; 88: 1937-42.
6. Barr E, Carroll J, Kalynych AM, *et al.* Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. Gene Ther 1994; 1: 51-8.
7. French BA, Mazur W, Geske RS, Bolli R. Direct *in vivo* gene transfer into porcine myocardium using replication-deficient adenoviral vectors. Circulation 1994; 90: 2414-24.
8. Dzau VJ, Morishita R, Gibbons GH. Gene therapy in the cardiovascular diseases. Trends Biotechnol 1993; 11: 205-10.
9. Morishita R, Gibbons GH, Dzau VJ. Gene therapy as potential treatment for cardiovascular diseases. In: Singh BN, ed. Cardiovascular Pharmacology and Therapeutics. New York: Livingstone, 1993; 51-61.
10. Aoki M, Morishita R, Muraishi A, *et al.* Efficient *in vivo* gene transfer into the heart in the rat myocardial infarction model using the HVJ (Hemagglutinating Virus of Japan)--liposome method. J Mol Cell Cardiol 1997; 29: 949-59.
11. Morishita R, Gibbons GH, Ellison KE, *et al.* Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. Proc Natl Acad Sci USA 1993; 90: 8474-8.
12. Morishita R, Gibbons GH, Ellison KE, *et al.* Intimal hyperplasia after vascular injury is inhibited by antisense cdk 2 kinase oligonucleotides. J Clin Invest 1994; 93: 1458-64.
13. Morishita R, Gibbons GH, Kaneda Y, Ogihara T, Dzau VJ. Pharmacokinetics of antisense oligodeoxyribonucleotides (cyclin B1 and CDC 2 kinase) in the vessel wall *in vivo*: enhanced therapeutic utility for restenosis by HVJ-liposome delivery. Gene 1994; 149: 13-9.
14. Morishita R, Gibbons GH, Horiuchi M, *et al.* A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*. Proc Natl Acad Sci USA 1995; 92: 5855-9.
15. Kaneda Y, Iwai K, Uchida T. Introduction and expression of the human insulin gene in adult rat liver. J Biol Chem 1989; 264: 12126-9.
16. von der Leyen HE, Gibbons GH, Morishita R, *et al.* Gene therapy inhibiting neointimal vascular lesion: *in vivo* transfer of endothelial cell nitric oxide synthase gene. Proc Natl Acad Sci USA 1995; 92: 1137-41.
17. Morishita R, Gibbons GH, Ellison KE, *et al.* Antisense oligonucleotides directed at cell cycle regulatory genes as strategy for restenosis therapy. Trans Assoc Am Physicians 1993; 106: 54-61.
18. Tomita N, Higaki J, Morishita R, *et al.* Direct *in vivo* gene introduction into rat kidney. Biochem Biophys Res Commun 1992; 186: 129-34.

19. Morishita R, Gibbons GH, Ellison KE, *et al.* Evidence for direct local effect of angiotensin in vascular hypertrophy. *In vivo* gene transfer of angiotensin converting enzyme. J Clin Invest 1994; 94: 978-84.
20. Kato K, Kaneda Y, Sakurai M, Nakanishi M, Okada Y. Direct injection of hepatitis B virus DNA into liver induced hepatitis in adult rats. J Biol Chem. 1991; 266: 22071-4.
21. Kaneda Y, Iwai K, Uchida T. Increased expression of DNA cointroduced with nuclear protein in adult rat liver. Science 1989; 243: 375-8.
22. Nakanishi M, Uchida T, Sugawa H, Ishiura M, Okada Y. Efficient introduction of contents of liposomes into cells using HVJ (Sendai virus). Exp Cell Res 1985; 159: 399-409.
23. Dzau VJ, Mann MJ, Morishita R, Kaneda Y. Fusigenic viral liposome for gene therapy in cardiovascular diseases. Proc Natl Acad Sci USA 1996; 93: 11421-5.
24. Acsadi G, Jiao SS, Jani A, *et al.* Direct gene transfer and expression into rat heart *in vivo*. New Biol 1991; 3: 71-81.
25. Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA. Behavior of genes directly injected into the rat heart *in vivo*. Circ Res 1992; 70: 193-8.
26. Gal D, Weir L, Leclerc G, Pickering JG, Hogan J, Isner JM. Direct myocardial transfection in two animal models. Evaluation of parameters affecting gene expression and percutaneous gene delivery. Lab Invest 1993; 68: 18-25.
27. Kitsis RN, Buttrick PM, McNally EM, Kaplan ML, Leinwand LA. Hormonal modulation of a gene injected into rat heart *in vivo*. Proc Natl Acad Sci USA. 1991; 88: 4138-42.
28. Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM. Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA. Circulation 1990; 82: 2217-21.
29. von Harsdorf R, Schott RJ, Shen YT, Vatner SF, Mahdavi V, Nadal-Ginard B. Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals. Circ Res 1993; 72: 688-95.
30. Kawaguchi H, Shin WS, Wang Y, *et al. In vivo* gene transfection of human endothelial cell nitric oxide synthase in cardiomyocytes causes apoptosis-like cell death. Identification using Sendai virus-coated liposomes. Circulation 1997; 95: 2441-7.
31. Kawada T, Nakatsuru Y, Sakamoto A, *et al.* Precise identification of gene products in hearts after *in vivo* gene transfection, using Sendai virus-coated proteoliposomes. Biochem Biophys Res Commun 1999; 259: 408-13.
32. French BA, Mazur W, Ali NM, *et al.* Percutaneous transluminal *in vivo* gene transfer by recombinant adenovirus in normal porcine coronary arteries, atherosclerotic arteries, and two models of coronary restenosis. Circulation 1994; 90: 2402-13.
33. Lemarchand PL, Jones M, Yamada I, Crystal RG. *In vivo* gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. Circ Res 1993; 72: 1132-8.
34. Ellison KE, Bishopric NH, Webster KA, *et al.* Fusigenic liposome-mediated DNA transfer into cardiac myocytes. J Mol Cell Cardiol 1996; 28: 1385-99.
35. Sawa Y, Suzuki K, Bai HZ, *et al.* Efficiency of *in vivo* gene transfection into transplanted rat heart by coronary infusion of HVJ liposome. Circulation 1995; 92(Suppl): II479-82.

EXHIBIT G

Reduced cardiac hypertrophy and altered blood pressure control in transgenic rats with the human tissue kallikrein gene[1]

JOSÉ-A. SILVA, JR.,*,† RONALDO C. ARAUJO,*,† OVIDIU BALTATU,* SUZANA M. OLIVEIRA,*,† CARSTEN TSCHÖPE,‡ EDWIN FINK,§ SIGRID HOFFMANN,* RALPH PLEHM,* KARL X. CHAI,¶ LEE CHAO,¶ JULIE CHAO,¶ DETLEV GANTEN*,†† JOÃO B. PESQUERO[1,2] AND MICHAEL BADER*,[2]

*Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany; †Department of Biophysics, Escola Paulista de Medicina, São Paulo, Brazil; ‡Departments of Cardiology and ††Clinical Pharmacology, University Hospital Benjamin Franklin, Free University, Berlin, Germany; §Department of Clinical Chemistry and Clinical Biochemistry, University of Munich, Germany; and ¶Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425, USA

SPECIFIC AIM

The aim of this study was to evaluate the cardiovascular actions of kinins, which have been implicated in the beneficial effects of angiotensin-converting enzyme (ACE) inhibitors by the generation and analysis of a transgenic rat line harboring the human tissue kallikrein gene, TGR(hKLK1).

PRINCIPAL FINDINGS

1. Transgene expression

Expression of a human tissue kallikrein transgene (hKLK1) under the control of the mouse metallothionein promoter was detected in all organs of the newly generated transgenic rat line, TGR(hKLK1) (**Fig. 1**). Translation of the hKLK1-mRNA was verified by the demonstration of human kallikrein in the urine of transgenic rats (700 ± 127 ng/ml).

2. Blood pressure

Mean arterial pressure determined by telemetric measurement turned out to be slightly but significantly lower in TGR(hKLK1) animals compared to Sprague Dawley (SD) control rats (110.5±1.1 vs. 114.9±1.0 mmHg; $P<0.01$). In contrast, no significant difference was observed between the transgenic and SD rats with respect to heart rate or locomotor activity. The B2 antagonist icatibant increased blood pressure (BP) significantly by 2.0 ± 0.9 mmHg ($P<0.01$) only in TGR(hKLK1).

The 24 h rhythm of mean arterial pressure in TGR(hKLK1) animals was dampened in comparison to untreated SD rats (amplitude of the dominant 24 h period in TGR(hKLK1): 1.6±0.2; in SD: 2.7±0.4; $P<0.05$). However, in both strains, the acrophases of the 24 h period occurred around midnight for BP, and the rhythms of heart rate and locomotor activity were similar.

3. Reduced cardiac hypertrophy and fibrosis in TGR(hKLK1)

To study the role of kallikrein in cardiac hypertrophy and fibrosis, TGR(hKLK1) and SD control rats were treated with a suppressor dose of isoproterenol for 7 days. This treatment resulted in a marked increase in relative heart (**Fig. 2***A*) and left ventricular weight (Fig. 2*B*) in both strains of rats. However, the effect was significantly less pronounced in TGR(hKLK1), indicating a protective action of transgene expression. Expression of ANP in the left ventricle, an early marker of cardiac hypertrophy, supported these findings. Whereas it was markedly induced in SD rats, no effect on ANP expression was observed in TGR(hKLK1) (Fig. 2*C*). Kinins and their B2 receptors obviously mediated the kallikrein action as the transgene effect on cardiac hypertrophy was abolished by the coadministration of icatibant (Fig. 2).

Moreover, interstitial fibrosis in the left ventricle, also induced by isoproterenol treatment and quantified by detecting collagen III mRNA in left ventricles, was less pronounced in TGR(hKLK1) compared to SD rats (Fig. 2*D*).

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.99-1010fje

[2] Correspondence: Max-Delbrück-Center for Molecular Medicine (MDC), Robert-Rössle-Strasse 10, D-13092 Berlin-Buch, Germany. E-mail: mbader@mdc-berlin.de

0892-6638/00/0014-1858/$02.25 © FASEB

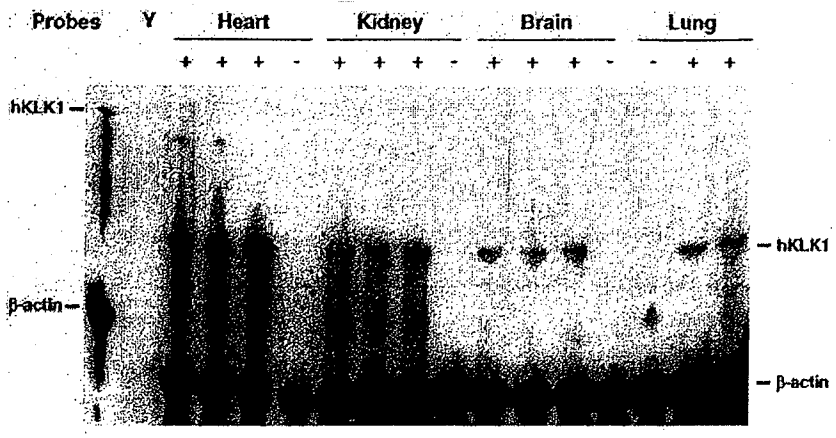


Figure 1. Ribonuclease protection assay detects expression of the hKLK1 gene in different organs of TGR(hKLK1) (+) but not in SD control rats (−). 20 μg of total RNA from tissues of individual animals or yeast (Y) together with hKLK1- and β-actin-specific probes was used.

CONCLUSIONS AND SIGNIFICANCE

Transgenic rats expressing human tissue kallikrein at high levels in all organs investigated became hypotensive probably through increased generation of kinins as evidenced by a partial normalization of BP after treatment with the B2 receptor antagonist icatibant. These findings are in line with results obtained in mice expressing the same transgene and hypertensive rats after somatic gene transfer of the hKLK1 gene.

An important novel observation in this study is the role of the kallikrein-kinin system (KKS) in circadian fluctuations of arterial pressure. The biological mechanisms underlying regulation of circadian rhythms of cardiovascular parameters are largely unknown. In mammals, most circadian rhythms are governed by what appears to be the main internal oscillator, the suprachiasmatic nucleus of the hypothalamus (SCN), since lesions in this brain region can obliterate the rhythms of heart rate and BP. Nonetheless, little is known about how oscillations in the SCN are biochemically achieved or which neuronal or hormonal pathways are used to establish the

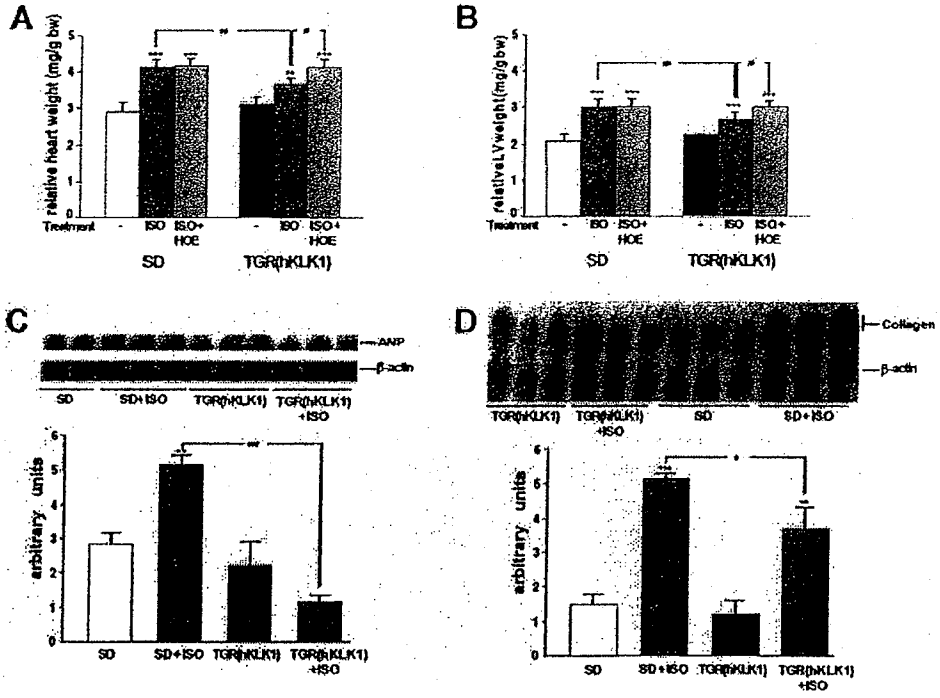
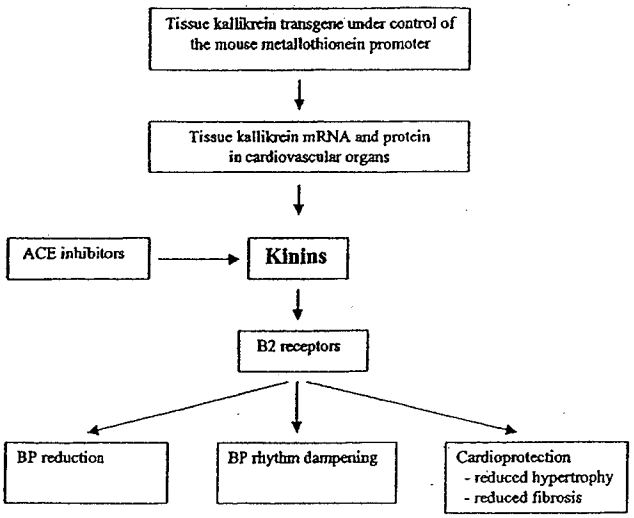


Figure 2. Cardiac hypertrophy and fibrosis after isoproterenol treatment is reduced in TGR(hKLK1) compared to SD rats. Heart weight (*A*) and left ventricular weight (*B*) to body weight ratios were determined of untreated SD and TGR(hKLK1) rats and of animals treated with isoproterenol (ISO) alone for 7 days (n=10) or with coadministration of icatibant (HOE, n=4). ANP (marker of hypertrophy, *C*) and collagen III (marker of fibrosis, *D*) expression were detected by ribonuclease protection assay in left ventricles of untreated and ISO-treated SD and TGR(hKLK1) rats (upper panel). 20 μg of total RNA of left ventricles was used with probes for rat ANP, collagen III, and β-actin. The levels of ANP and collagen III mRNA relative to β-actin mRNA were quantified using the software TINA on a PhosphorImager system (lower panel). $^{***}P < 0.005$, $^{**}P < 0.01$ vs untreated rats; $^{\#\#\#}P < 0.005$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$.



Scheme 1

rhythm of the cardiovascular system. Kallikrein, kininogens, kinins, and their receptors, B1 and B2, are present in several regions of the brain, including SCN, and the central KKS is involved in BP regulation. Furthermore, a circadian rhythm of kallikrein expression has recently been reported in the rat pineal gland. Kinins may also be important in transmitting the oscillations generated in SCN and pineal gland to other brain areas or to the periphery. A peripheral action of the KKS governing diurnal BP variations is supported by the circadian rhythm of urinary kallikrein excretion reported, with highest concentrations preceding lowest BP values. Thus, the KKS may be deeply involved in the circadian regulation of BP. As the circadian rhythmicity of heart rate is unaltered in TGR(hKLK1), this study confirms earlier reports that the rhythms of BP and heart rate are differentially regulated.

The most important novel finding of this study is the prohibitive effect of tissue kallikrein in isoproterenol-induced cardiac hypertrophy and fibrosis. Reductions in hypertrophy and fibrosis were abolished by icatibant, which further supports kinins as mediators of the transgene effects. Consistent with an important role of kinins, previous pharmacological studies have shown that isoproterenol-induced cardiac hypertrophy can be diminished more effectively by ACE inhibitors than by AT1 antagonists. Kinins have also been implicated in the antihypertrophic actions of ACE inhibitors in the aortic coarctation model since icatibant effectively blunted the drug-induced reduction of left ventricular hypertrophy. Furthermore, bradykinin infusion prevented development of increased cardiac mass in the same model. While kinins may act as weak growth factors on cultured cardiomyocytes and fibroblasts, they have also been shown to reduce cardiac collagen synthesis and growth via release of prostaglandins and nitric oxide. According to our results, the latter seems to be the prevailing *in vivo* effect. Adaptation of cardiac muscle to an increased work load can be achieved either by an increase in muscular mass (i.e., hypertrophy) or by an improved performance of the existing myocardium. Kinins may inhibit hypertrophy by improving the supply of cardiomyocytes with nutrients and oxygen via two independent mechanisms: increase in coronary perfusion and stimulation of myocardial glucose uptake. These kinin effects may be crucial for the prominent cardioprotective actions of ACE inhibitors. In summary, we have developed a new transgenic rat model with an overactive KKS. These rats may be applicable to the study of multiple issues of cardiovascular regulation and other physiological and pathophysiological mechanisms in which kinins might participate. FJ

This work was supported by grants from the European Union (Transgeneur), the Deutscher Akademischer Austauschdienst (PROBRAL), the Fundação de Amparo à Pasquisa do Estado de São Paulo (FAPESP), and the Volkswagenstiftung. R.C.A. and J.B.P. received fellowships from the Max-Delbrück Center for Molecular Medicine and J.A.S. from FAPESP. We thank Dr. K. Wirth (Hoechst Marion Roussel) for his generous gift of icatibant and Dr. X. Tian for the collagen III probe. The excellent technical assistance of M. Somnitz and A. Böttger is gratefully acknowledged.

EXHIBIT H

Am J Physiol Heart Circ Physiol
279: H986–H991, 2000.

Preservation of myocardial function after adenoviral gene transfer in isolated myocardium

S. E. LEHNART,[1] P. M. L. JANSSEN,[1] W. M. FRANZ,[2] J. K. DONAHUE,[3] J. H. LAWRENCE,[3] E. MARBÁN,[3] J. PRESTLE,[1] AND G. HASENFUSS[1]
[1]*Abteilung Kardiologie und Pneumologie, Universität Göttingen, D-37075 Göttingen, Germany;* [2]*Medizinische Klinik II, Universität zu Lübeck, D-23538 Lübeck, Germany; and* [3]*Section of Molecular and Cellular Cardiology, Johns Hopkins University Medical School, Baltimore, Maryland 21205*

Received 8 July 1999; accepted in final form 11 February 2000

Lehnart, S. E., P. M. L. Janssen, W. M. Franz, J. K. Donahue, J. H. Lawrence, E. Marbán, J. Prestle, and G. Hasenfuss. Preservation of myocardial function after adenoviral gene transfer in isolated myocardium. *Am J Physiol Heart Circ Physiol* 279: H986–H991, 2000.—Adenoviral gene transfer to the heart represents a promising model for structure-function analyses. Rabbit hearts were subjected to an ex vivo perfusion protocol that achieves gene transfer in >90% of cardiac myocytes. Contractile function of isolated myocardial preparations of these hearts was then observed for 2 days in a recently developed trabecula culture system. In sham-infected hearts, the initial developed force (F_{init}) (15.6 ± 3.7 mN/mm^2; n = 12) did not change significantly after 48 h (17.0 ± 1.9 mN/mm^2; P = 0.46). In adenovirus-infected preparations, F_{init} (14.3 ± 1.8 mN/mm^2; n = 21) did not significantly differ from the control (P = 0.75) and was unchanged after 48 h (15.3 ± 2.5 mN/mm^2; P = 0.93). After 2 days of continuous contractions, we observed homogenous and high-level expression of the reporter genes *LacZ* coding for β-galactosidase and *Luc* coding for firefly luciferase. Luciferase activity increased more than 2,500-fold from background levels of 8.7 × 10^3 ± 5.0 × 10^3 relative light units (RLU)/mg protein (from hearts transfected with promotorless adenovirus with luciferase transgene construct AdNULLLuc, n = 5) to 23.4 × 10^6 ± 11.1 × 10^6 RLU/mg protein (from hearts tranfected with adenovirus with Rous sarcoma virus promotor and luciferase transgene construct AdRSVLuc, n = 5) in infected myocardial preparations (P < 0.005). Our results demonstrate a new ex vivo approach to achieve homogenous and high-level expression of recombinant adenoviral genes in contracting myocardium without adverse functional effects.

adenovirus; trabecula; rabbit

GENE TRANSFER TO THE HEART for the replacement of missing cellular proteins or for the expression of therapeutic gene products represents a promising approach for the treatment of myocardial disorders (5, 17, 22, 24, 26). Previous studies (1, 12, 16, 27) have demonstrated the feasibility of recombinant adenoviruses as gene delivery systems to improve cardiac function on a cellular level by expression of gene products considered to be relevant to the failing heart, e.g., adenoviral transfer of a potassium channel gene increases K^+ currents and shortens action potential duration in failing cardiac myocytes (27), and overexpression of the sarcoplasmic (endo)reticulum Ca^{2+}-ATPase in cardiac myocytes enhances sarcoplasmic reticulum Ca^{2+}-uptake and shortens prolonged intracellular Ca^{2+} transients (12).

The intact heart muscle represents a much higher burden in terms of effective gene transfer to tissue-bound cardiac myocytes and in the study of consecutive functional effects. Few studies have been able to show effective expression of adenovirally introduced proteins in the working heart (16, 23), and functional studies of the heart after adenoviral infection under in vivo conditions have been limited by strong immunogenicity and unknown intrinsic toxicity of the vector system against the host cell (11, 31). Therefore, it might be difficult to study functional implications of adenoviral gene transfer under in vivo conditions. On the other hand, the functional study of adenovirus-infected cultured cardiac myocytes, thereby avoiding an immunologic response, is limited by their progressive dedifferentiation over the expression time (6) and by technical difficulties to qualitatively evaluate and to extrapolate the contractile function of isolated cells to the more physiological multicellular myocardial architecture accomplishing loaded contractions. Therefore, multiday observation of adenoviral-infected isolated myocardial preparations would allow us to identify adverse functional effects caused by intrinsic toxicity of one of the most efficient gene transfer vehicles. To achieve this goal, we established a new technique to study consequences of adenoviral transgene expression on myocardial function in a multicellular muscle preparation under well-defined physiological conditions. The technique is based on a recently developed trabecula culture system (20) and a highly efficient adenoviral gene transfer protocol (8, 9) and shows that con-

Address for reprint requests and other correspondence: S. E. Lehnart, Abt. Kardiologie und Pneumologie, Universität Göttingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany (E-mail: slehnart@med.uni-goettingen.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

tractile function is not altered by adenoviral gene transfer per se.

MATERIALS AND METHODS

Preparation and ex vivo transfection of rabbit hearts. Female New Zealand White rabbits (1.5–2.5 kg) received heparin anticoagulation (1,000 U iv) before pentobarbital sodium anesthesia (Nembutal, 60 mg/kg iv). Under sterile conditions, the hearts were rapidly excised and rinsed twice in ice-cold, HEPES-modified Krebs-Henseleit (K-H) buffer containing (in mM) 138.2 Na^+, 5.0 K^+, 1.2 Mg^{2+}, 1.0 Ca^{2+}, 144.4 Cl^-, 1.2 SO_4^{2-}, 1.2 $H_2PO_4^-$, 20.0 HEPES, and 15.0 glucose, saturated with O_2 at pH 7.4. Hearts were mounted and suspended in an insulated chamber at 35–37°C and were retrogradely perfused via the ascending aorta by a modified Langendorff-perfusion technique (8) with oxygenated K-H buffer for 5 min at 30–40 ml/min; this was followed by 25 ml of oxygenated K-H buffer containing 1 mg/ml albumin and 1.6×10^9 plaque-forming units (pfu)/ml of recombinant adenovirus. Higher virus concentrations could not be used without compromising the myocardial viability during the perfusion protocol, which would occur due to the altered composition of solutions used in our experiments if more virus was added. The virus-containing perfusate was recirculated for 60 min at 37°C with a controlled flow rate of 30 ml/min. After the infection protocol, the hearts were perfused with virus-free K-H buffer to wash out the virus (for at least 5 min). The high transfection efficiency of this perfusion protocol (over 90% transfected cardiac myocytes) was tested at regular intervals by quantifying the percentage of cells expressing β-galactosidase (β-Gal) by evaluation of primary cultures of cardiac myocytes of AdCMVLacZ (adenovirus-transfected human cytomegalovirus-promoted *LacZ* transgene construct)-infected hearts (8, 9). As a control, hearts were sham infected using the same protocol without viral vectors or with AdNULLLuc (adenovirus vector with promotorless luciferase gene) for 60 min. All procedures and protocols were approved by the local Animal Care and Use Committee in accordance with institutional guidelines.

Adenovirus vectors. Three types of first-generation human type 5 recombinant adenoviruses (Ad) were used: AdCMVLacZ, encoding *Escherichia coli* β-Gal under control of the human cytomegalovirus (CMV) immediate early promoter, and two different adenoviral luciferase constructs containing the firefly luciferase gene in the presence or the absence of the Rous sarcoma virus (RSV) long terminal repeat promotor (AdRSVLuc and AdNULLLuc, respectively) (10). High-titer adenovirus stocks were prepared and tested for replication-competent adenoviruses as described (7, 13). Adenoviral titers were determined by averaging two plaque titration assays on HEK 293 cells as described previously (8). Although the current adenoviral vectors might have limitations in effectiveness in "long-term" expression (months/years), the fast and robust expression makes them especially useful in these "short-term" expression experiments (days/weeks).

Myocardial trabecula dissection. To dissect thin, uniform myocardial trabeculae and small papillary muscles from the free wall and septum of the right ventricle, hearts were additionally perfused with K-H solution containing (in mM) 141.2 Na^+, 5.0 K^+, 2.0 Mg^{2+}, 1.0 Ca^{2+}, 127 Cl^-, 2.0 SO_4^{2-}, 1.2 $H_2PO_4^-$, 20.0 HCO_3^-, 10.0 glucose, and 20.0 2,3-butanedione monoxime (BDM) as a cardioprotective ingredient (25) in equilibrium with 95% O_2-5% CO_2. After spontaneous beating of the suspended heart had stopped, preparations were carefully dissected under a binocular dissecting microscope (19, 28).

Trabecula culture system. Preparations were mounted in a closed sterile chamber (Scientific Instruments, Heidelberg, Germany) between the basket-shaped extension of a force transducer and the hooklike extension of a micromanipulator screw (20). The BDM-containing solution was exchanged under sterile conditions for BDM-free K-H solution, and the extracellular Ca^{2+} concentration was increased stepwise to 2.0 mmol/l. The solution was then exchanged for 199 cell culture medium (Sigma) equilibrated with 95% O_2-5% CO_2 containing the following modifications (in mM): 2.0 DL-carnitine, 5.0 creatine, 5.0 taurine, and 2.0 L-glutamine as well as 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 20 IU/l human insulin (Opti-Pen, Hoechst, Germany). The muscles were stimulated at ~30% over a threshold voltage (2–4 V) through 5-ms asymmetric pulses at a frequency of 0.5 Hz. The muscles were carefully stretched to the length at which passive force development was ~2–10% of active developed force at 1.75 mM Ca^{2+}, reflecting a sarcomere length between 2.1 and 2.2 μm (28). After force had stabilized, online data collection was started. All experiments were executed under isometric conditions for 48 h at a 0.5-Hz stimulation frequency at 37°C with a pH of 7.4. For more detailed description of the setup and procedures, see Janssen et al. (20). After 48 h of continuous contractions, 1 μM isoproterenol was added to test for β-adrenergic response and the presence of contractile reserve of these preparations.

Reporter gene assays. Forty-eight hours after adenoviral infection and continuous monitoring of contractile function in the trabecula culture system, the myocardial preparations were evaluated qualitatively for β-Gal expression by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or quantitatively for luciferase activity by chemoluminescent assay. Myocardial preparations that were infected with AdCMVLacZ were fixed in 0.1% glutaraldehyde in PBS for 20 min at room temperature, washed with PBS, and then stained with (in mM) 15.0 $K_4Fe(CN)_6$, 15.0 $K_3Fe(CN)_6$, and 1.0 $MgCl_2$ and 1 mg/ml X-Gal in PBS at 37°C. The staining solution was aspirated, and the trabeculae were permanently fixed in 1% glutaraldehyde in PBS. Even though we did not detect any endogenous β-Gal activity in control experiments with nontransfected hearts, staining of the myocardial preparations was performed at alkaline conditions (pH 8.5) to avoid false-positive detection of endogenous mammalian β-Gal activity (30). Thicker preparations (>350 μm) were sometimes divided and restained to overcome the diffusion limitations of X-Gal. After 12 h, the X-Gal solution was removed, and the trabeculae were evaluated microscopically for blue staining, embedded in paraffin, sectioned at 15 μm, mounted on glass slides, and then counterstained with hematoxylin and eosin. Histological sections were evaluated for diffuse β-Gal activity at ×40 magnification. Because the intense blue staining diminished individual cell borders, we were not able to quantitatively give the average percentage of stained cardiac myocytes in the multicellular preparation.

Alternatively, myocardial trabeculae were infected with replication-defective adenoviruses encoding firefly luciferase. The promotorless construct AdNULLLuc served as a negative control for background activity determination. Alternatively, the *Luc* transgene was under the control of the RSV promotor for maximal *Luc* expression (10). After 48 h of continuous contractions, myocardial tissue was frozen in liquid nitrogen. Myocardial preparations were homogenized in lysis buffer (1% vol/vol Triton X-100, 1 mM 1,4-dithiothreitol, and 100 mM potassium phosphate, pH 7.8) and centri-

fuged, and the supernatant was used to perform luciferase assays (10). All tissue luciferase recordings were normalized for protein concentration as determined by the Bradford assay. Luciferase activity is given in relative light units (RLU) per milligram protein after correction for background activity (10).

Data analysis and statistics. For a period of at least 48 h, twitch contractions of myocardial trabeculae were monitored online by a data acquisition and analysis program written in LabView (National Instruments; 1-kHz sample frequency). The following contractile parameters were analyzed: peak developed force (F_{dev}; mN/mm^2), diastolic force (F_{diast}; mN/mm^2), time from stimulation to peak force (TTP; ms), maximum relative rate of force development [$(dF/dt_{max})/F$; s^{-1}], maximum relative rate of force decline [$(dF/dt_{min})/F$; s^{-1}], time from peak force to 50% relaxation (RT_{50}; in ms), and times from stimulation to 50% and 90% relaxation (TT_{50} and TT_{90}, respectively; in ms). The initial developed force (F_{init}; mN/mm^2) was calculated as the average developed force during the first hour of stimulation. Preparations that displayed rapid rundown (>40%) of F_{dev} during the first 3 h or in which F_{init} was <5 mN/mm^2 were discarded from further study. Unless otherwise stated, all experiments are presented as means ± SE.

Force and twitch timing data were analyzed using two-factor repeated measures ANOVA: we tested 33 subjects (muscles), with (virus) factors (control, *LacZ*, and *Luc*) and time (0 and 48 h). This test design detects any significant differences between 0 and 48 h, within each group, among the viral factors, and in the interaction between virus and time. Statistical significance was determined by Student's *t*-test for paired or unpaired data where applicable. $P < 0.05$ was accepted as significant.

RESULTS

Basic characteristics of contraction and relaxation. Myocardial preparations were subdivided into three groups according to the infection protocols: sham-infected preparations (n = 12) served as the control group and were compared with the intervention groups *LacZ* (n = 11) and *Luc* (n = 10). The average dimensions of the preparations were the following: length, 3.85 ± 0.19 mm; thickness, 363 ± 27 μm; width, 412 ± 23 μm; and cross-sectional area, 0.128 ± 0.013 mm^2. Average size and distribution of the preparation dimensions were similar in all groups. In pilot experiments we found no effect of the perfusion protocol per se on contractile function. The contraction parameters behaved similarly over a 48-h period under identical conditions with this study and without Langendorff perfusion (20). At 0.5 Hz stimulation frequency and 37°C, the F_{init} was 15.6 ± 3.7 mN/mm^2 in the control group, 14.2 ± 2.3 mN/mm^2 in the *Luc* group (unpaired *t*-test, P = 0.75), and 14.3 ± 2.8 mN/mm^2 in the *LacZ* group (P = 0.77). Also, there were no significant differences in the diastolic force (F_{diast}) at t = 0 h for the control group (1.64 ± 0.45 mN/mm^2), the *Luc* group (1.05 ± 0.21 mN/mm^2, P = 0.26), and the *LacZ* group (1.21 ± 0.24 mN/mm^2, P = 0.41) or between the *LacZ* group and the *Luc* group (P = 0.62). ANOVA indicated no changes in contractile parameters F_{dev} and F_{diast} with respect to the variables time (0 and 48 h; F_{dev}: P = 0.97 and F_{diast}: P = 0.55) and virus (control, *LacZ*, and *Luc* groups; F_{dev}: P = 0.90 and F_{diast}: P = 0.52) or between the interaction of these factors (time × virus; F_{dev}: P = 0.99 and F_{diast}: P = 0.94). Furthermore, there was no significant difference in the number of preparations discarded for further study between the control and either of the gene transfer groups. In total, 7 (4 *Luc*, 2 sham, and 1 *LacZ*) of 40 preparations failed to qualify for further study. Both sham- and *LacZ*- or *Luc*-transfected preparations did not show contractile abnormalities over the time course of 48 h.

Influence of transgene expression on myocardial performance. Sham-transfected myocardial trabeculae contracted continuously over 48 h and did not show alterations of contractile parameters, e.g., F_{dev} at t = 48 h was 17.0 ± 1.9 mN/mm^2 compared with 15.6 ± 3.7 mN/mm^2 at t = 0 h (P = 0.46; Fig. 1). After a 2-day time span, neither *LacZ*-transfected (P = 0.31) nor *Luc*-transfected (P = 0.06) myocardial preparations displayed a significant change from t = 0 h (ANOVA; time, P = 0.55; virus, P = 0.09; time × virus, P = 0.94) in the change in RT_{50}, despite robust expression of the reporter genes (Fig. 2). Similarly, there were no significant differences between the groups for other twitch timing parameters (TTP, TT_{50}, and TT_{90}). Additionally, there were no significant differences within the control group, the *LacZ* group, or the *Luc* group regarding $(dF/dt_{max})/F$ or $(dF/dt_{min})/F$ over 48 h, and there were no significant differences between the groups (Fig. 3).

Effects of reporter gene expression on β-adrenergic signaling. Inotropic stimulation of the heart via β-adrenergic receptors represents the most powerful way to

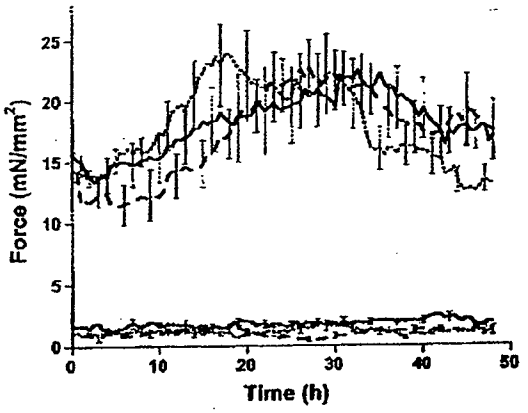


Fig. 1. Average time courses of peak force (F_{dev}; *top lines*) and diastolic force development (F_{diast}; *bottom lines*) over 48 h of the control group [promotorless adenovirus with luciferase transgene construct (AdNULLLuc)] (solid lines), the *LacZ* [adenovirus with human cytomegalovirus promotor and *LacZ* transgene (AdCMVLacZ)]-transfected group (dotted lines), and the *Luc* [adenovirus with Rous sarcoma virus promotor and luciferase transgene construct (AdRSVLuc)]-transfected group (dashed lines). The *Luc* transgene codes for luciferase and the *LacZ* transgene codes for β-galactosidase (β-Gal). F_{dev} and F_{diast} were calculated as fractions of initial developed force; average normalized data were later multiplied by average force per cross-sectional area for display purposes. Changes in these parameters due to adenoviral gene expression were not significant over 48 h (control group, n = 11; *LacZ* group, n = 11; and *Luc* group, n = 10).

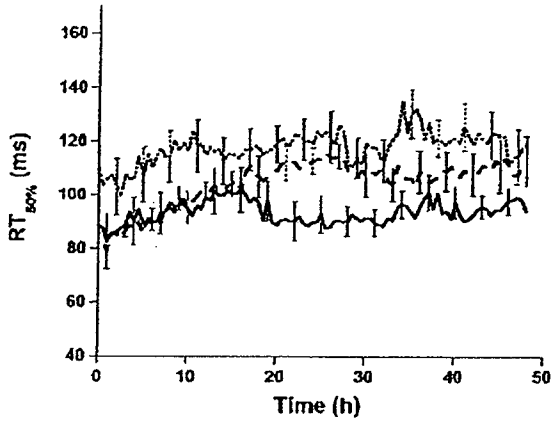


Fig. 2. Average time courses of time from peak force to 50% relaxation (RT_{50}) over 48 h of the control group (solid lines), the *LacZ*-transfected group (dotted lines), and the *Luc*-transfected group (dashed lines). No significant changes due to adenoviral gene expression were observed over 48 h (control group, $n = 11$; *LacZ* group, $n = 11$; and *Luc* group, $n = 10$).

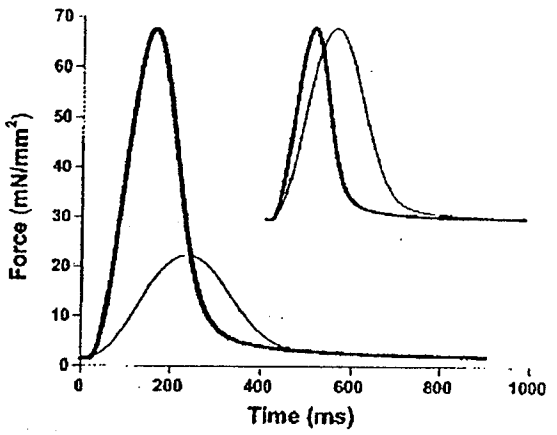


Fig. 4. Representative tracing from an AdRSVLuc-transfected myocardial preparation after 48 h of continuous contractions (thin line) and contractile response to 1 μM isoproterenol (thick line). Same tracings expressed as fractions of their individual peak developed tension to show absence of effect on twitch timing more clearly (*inset*). Control twitch was obtained 5 min before intervention, and intervention twitch was obtained after force had stabilized.

increase cardiac contractility. Global adenoviral infection followed by 48 h of sustained contractions did not significantly affect the β-adrenergic pathway. Figure 4 shows a representative experiment with 1 μM isoproterenol to stimulate a β-adrenergic response after 48 h of continuous contractions in a *Luc*-infected preparation. On average, maximal developed force in these myocardial preparations after exposure to isoproterenol increased to 351 ± 114% in *LacZ*-transfected ($n = 3$) and to 326 ± 91% in *Luc*-transfected preparations ($n = 3$) compared with 340 ± 104% in the control group ($n = 3$). Relaxation as reflected by RT_{50} was accelerated to 69 ± 2% in the *LacZ* group ($n = 3$), to 66 ± 6% in the *Luc* group ($n = 3$), and to 63 ± 4% in the control group ($n = 3$).

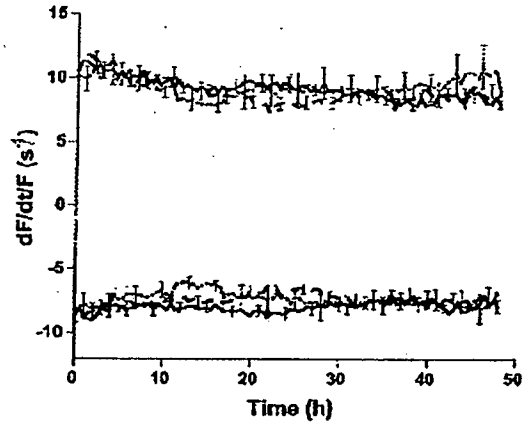


Fig. 3. Average time courses of maximum relative rate of force development [$(dF/dt_{max})/F$] (*top lines*) and decline [$(dF/dt_{min})/F$] (*bottom lines*) over 48 h of the control group (solid lines), the *LacZ*-transfected group (dotted lines), and the *Luc*-transfected group (dashed lines). No significant changes in these parameters were observed over 48 h.

Expression pattern of LacZ in myocardial trabeculae. Myocardial preparations that were subjected to *LacZ* transfection and kept contracting continuously for 48 h were fixed and stained for β-Gal expression ($n = 11$). As shown in Fig. 5, there is widespread and robust expression of the *LacZ* transgene, with nearly all cardiac myocytes staining positive. Sham-infected preparations or those infected with the promotorless *Luc* transgene adenovirus demonstrated no X-Gal staining.

Myocardial expression of luciferase. Forty-eight hours after infection with 1.6×10^9 pfu/ml of AdRSVLuc and continuous stimulation, myocardial preparations showed high levels of luciferase activity. Myocardial preparations that were infected with AdNULLLuc served as background levels and had an average luciferase activity of $8.7 \times 10^3 \pm 5.0 \times 10^3$ RLU/mg protein. In sharp contrast, the constitutive RSV promoter served for a significant increase in luciferase activity to $23.4 \times 10^6 \pm 11.1 \times 10^6$ RLU/mg protein in transfected myocardial preparations, being more than 2,500-fold higher than control levels ($n = 5$; $P < 0.005$).

DISCUSSION

In light of upcoming gene therapy protocols in the future, we set out to directly demonstrate a lack of adverse effects on myocardial function despite robust overexpression of adenoviral transgenes. The present study demonstrates highly effective gene transfer to multicellular myocardial preparations without adverse influences on contractile function. This was accomplished by combining a recently developed myocardial trabecula culture system (20) with an ex vivo gene transfer technique (8, 9). Under physiological conditions and continuous monitoring, contractile performance was stable in infected preparations and was not significantly different from control preparations throughout this period. Neither the perfusion protocol

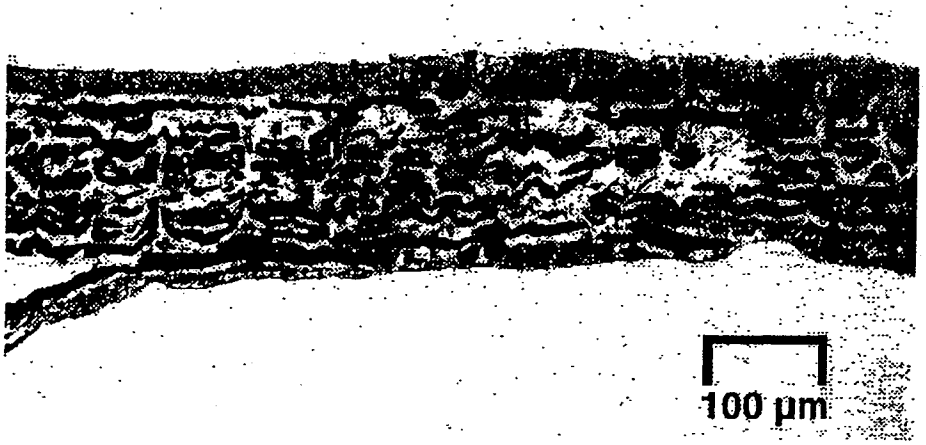


Fig. 5. Histological detection of β-Gal activity in myocardium 2 days after perfusion with 1.6×10^9 plaque-forming units/ml of AdCMVLacZ followed by continuous contractions. Photomicrograph of a longitudinal histological section through a myocardial preparation demonstrating homogenous distribution of the *LacZ* gene product throughout the myocardial tissue. Dimensions of this preparation before fixation and staining were the following: width, 200 μm; thickness, 150 μm; and length, 3.0 mm.

nor adenovirus infection and reporter gene expression adversely influence contractile function of these multicellular preparations. Thus, by use of an adenoviral perfusion protocol in intact hearts, it is possible to induce high levels of transgene expression into myocardial preparations without inducing adverse effects by toxicity of the adenoviral vector system on myocardial function.

Previous studies investigating the influence of transgenes on myocardial function have used cardiac myocytes under primary culture conditions as a model to study adenoviral gene transfer and subsequent effects of recombinant proteins on contractility (1, 12, 22, 27). The culture of myocardial preparations used in this study has several advantages over the culture of isolated cardiac myocytes, because it represents *1*) the more complex situation of gene delivery into a differentiated multicellular architecture like the intact heart muscle (2, 8, 9, 11, 14, 16, 23, 29); *2*) the more physiological situation of loaded conditions in the study of contractile function (4, 20); and *3*) the stable contractile behavior, protein synthesis, and nondedifferentiating cellular integrity of electrically stimulated myocardial preparations (3, 4, 20). In addition, the recent demonstration of the long-term culture of human trabeculae for up to 6 days suggests the possibility of using this gene transfer protocol to investigate pathophysiological alterations in the failing human heart (19).

The functionally intact myocardial preparations from transfected hearts showed high-level expression of different reporter genes after 2 days. β-Gal expression was homogenous and robust within thin preparations, and myocardial luciferase activity was more than 2,500-fold higher than control levels. The functional effects of adenovirus infection and of reporter gene expression have been evaluated in a controlled preparation of intact myocardium. The absence of toxic effects of the adenoviral vector and the expressed reporter genes *LacZ* and *Luc* on contracile function is in close agreement with studies on isolated cardiac myocytes (15, 22).

The trabecula culture method as a technique for assessment of recombinant transgene expression on myocardial function may be an alternative to conventional transgenic animal models. Developmental adaption in transgenic animals to overexpression or knockout of a given protein often results in a new phenotype. This can be difficult to differentiate for the specific effects of the given transgene on myocardial function from secondary effects, e.g., transgenic animals overexpressing sarcoplasmic (endo)reticulum Ca^{2+}-ATPase (18) or calsequestrin (21) also exhibit increased transcription of other physiologically important proteins like phospholamban. Similarly, functionally meaningful overexpression in transgenic mice downregulates gene expression of Ca^{2+}-regulatory proteins like the ryanodine receptor, triadin, and junctin by 50% or more, whereas phospholamban and Ca^{2+}-ATPase are upregulated. Adenoviral gene transfer techniques may not only bypass developmental adaption, but they may also be used to knock out a target gene by programming antisense strategies that block gene expression, and they represent a future therapeutic approach under investigation.

In conclusion, adenoviral transfection of the heart by a well-defined and effective perfusion protocol does not affect myocardial function under physiological conditions. The technique used in this study achieves homogenous and high-level expression of the reporter genes *LacZ* and *Luc*. Continuous multiday monitoring of myocardial function under ex vivo conditions in the trabecula culture system during establishment of transgene expression showed no adverse functional effects due to possible intrinsic toxicity of the adenoviral vector system in cardiac myocytes. Adenoviral perfusion and infection of the myocardium therefore seems to be safe in terms of functional side effects. This

approach may help to investigate transgene effects in the heart by a structure-function analysis of recombinant proteins on the level of the contracting myocardium.

We gratefully acknowledge the excellent support from A. Janssen, K. Beulich, and U. Bieligk in cell culture and recombinant adenovirus techniques and Dr. C. Ihling and colleagues for help with the histology (all from the University of Freiburg, Germany).

The research presented in this paper was supported by a grant from the Deutsche Forschungsgemeinschaft (HA 1233/3-2).

REFERENCES

1. **Akhter SA, Skaer CA, Kypson AP, McDonald PH, Peppel KC, Glower DD, Lefkowitz RJ, and Koch WJ.** Restoration of β-adrenergic signaling in failing cardiac ventricular myocytes via adenoviral-mediated gene transfer. *Proc Natl Acad Sci USA* 94: 12100–12105, 1997.
2. **Barr E, Carroll J, Kalynych AM, Tripathy SK, Kozarsky K, Wilson JM, and Leiden JM.** Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. *Gene Ther* 1: 51–58, 1994.
3. **Berger HJ, Prasad SK, Davidoff AJ, Pimental D, Ellingsen O, Marsh JD, Smith TW, and Kelly RA.** Continual electric field stimulation preserves contractile function of adult ventricular myocytes in primary culture. *Am J Physiol Heart Circ Physiol* 266: H341–H349, 1994.
4. **Bers DM.** Ca^{2+} sparks. Jumping the gap from the cell to cardiac muscle. *Circ Res* 81: 636–638, 1997.
5. **Brodde OE, Michel MC, and Zerkowski HR.** Signal transduction mechanisms controlling cardiac contractility and their alterations in chronic heart failure. *Cardiovasc Res* 30: 570–584, 1995.
6. **Claycomb WC and Palazzo MC.** Culture of the terminally differentiated adult cardiac mucle cell: a light and scanning electron microscope study. *Dev Biol* 80: 466–482, 1980.
7. **Curiel DT, Agarwal S, Wagner E, and Cotten M.** Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc Natl Acad Sci USA* 88: 8850–8854, 1991.
8. **Donahue JK, Kikkawa K, Johns DC, Marbán E, and Lawrence JH.** Ultrarapid, highly efficient viral gene transfer to the heart. *Proc Natl Acad Sci USA* 94: 4664–4668, 1997.
9. **Donahue JK, Kikkawa K, Thomas AD, Marbán E, and Lawrence JH.** Acceleration of widespread adonoviral gene transfer to intact rabbit hearts by coronary perfusion with low calcium and serotonin. *Gene Ther* 5: 630–634, 1998.
10. **Franz WM, Rothmann T, Frey N, and Katus HA.** Analysis of tissue-specific gene delivery by recombinant adenoviruses containing cardiac-specific promotors. *Cardiovasc Res* 35: 560–566, 1997.
11. **French BA, Mazur W, Geske RS, and Bolli R.** Direct in vivo gene transfer into porcine myocardium using replication-deficient adenoviral vectors. *Circulation* 90: 2414–2424, 1994.
12. **Giordano FJ, He H, McDonough P, Meyer M, Sayen R, and Dillmann WH.** Adenovirus-mediated gene transfer reconstitutes depressed sarcoplasmic reticulum Ca^{2+}-ATPase levels and shortens prolonged cardiac myocyte Ca^{2+} transients. *Circulation* 96: 400–403, 1997.
13. **Graham FL and Prevec L.** Methods for construction of adenovirus vectors. *Mol Biotechnol* 3: 207–220, 1995.
14. **Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, and Finkel T.** Efficient gene transfer into myocardium by direct injection of adenovirus vectors. *Circ Res* 73: 1202–1207, 1993.
15. **Hajjar RJ, Schmidt U, Kang JX, Matsui T, and Rosenzweig A.** Adenoviral gene transfer of phospholamban in isolated rat cardiomyocytes. Rescue effects by concomitant gene transfer of sarcoplasmic reticulum Ca^{2+}-ATPase. *Circ Res* 81: 145–153, 1997.
16. **Hajjar RJ, Schmidt U, Matsui T, Guerrero JL, Lee KH, Gwathmey JK, Dec GW, Semigran MJ, and Rosenzweig A.** Modulation of ventricular function through gene transfer in vivo. *Proc Natl Acad Sci USA* 95: 5251–5256, 1998.
17. **Hasenfuss G.** Calcium pump overexpression and myocardial function. Implications for gene therapy of myocardial failure. *Circ Res* 83: 966–968, 1998.
18. **He H, Giordano FJ, Hilal-Dandan R, Choi DJ, Rockman HA, McDonough PM, Bluhm WF, Meyer M, Sayen MR, Swanson E, and Dillmann DW.** Overexpression of the rat sarcoplasmic reticulum Ca^{2+} ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. *J Clin Invest* 100: 380–389, 1997.
19. **Janssen PM, Lehnart SE, Prestle J, and Hasenfuss G.** Preservation of contractile characteristics of human myocardium in multi-day cell culture. *J Mol Cell Cardiol* 31: 1419–1427, 1999.
20. **Janssen PML, Lehnart SE, Prestle J, Lynker JC, Salfeld P, Just H, and Hasenfuss G.** The trabecula culture system: a novel technique to study contractile parameters over a multiday time period. *Am J Physiol Heart Circ Physiol* 274: H1481–H1488, 1998.
21. **Jones LR, Suzuki YJ, Wang W, Kobayashi YM, Ramesh V, Franzini-Armstrong C, Cleemann L, and Morad M.** Regulation of Ca^{2+} signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J Clin Invest* 101: 1385–1393, 1998.
22. **Kirshenbaum LA, Angelides KJ, and Schneider MD.** Detection of exogenous gene expression in live adult ventricular myocytes after adenoviral gene delivery. *Circulation* 90: 2124–2125, 1994.
23. **Kypson AP, Peppel K, Akhter SA, Lilly RE, Glower DD, Lefkowitz RJ, and Koch WJ.** Ex vivo adenovirus-mediated gene transfer to the adult rat heart. *J Thorac Cardiovasc Surg* 115: 623–630, 1998.
24. **Lehnart SE, Schillinger W, Pieske B, Prestle J, Just H, and Hasenfuss G.** Sarcoplasmic reticulum proteins in heart failure. *Ann NY Acad Sci* 853: 220–230, 1998.
25. **Mulieri LA, Hasenfuss G, Ittleman F, Blanchard EM, and Alpert NR.** Protection of left ventricular myocardium from cutting injury with 2,3-butanedione monoxime. *Circ Res* 65: 1441–1444, 1989.
26. **Näbauer M, and Kääb S.** Potassium channel down-regulation in heart failure. *Cardiovasc Res* 37: 324–334, 1998.
27. **Nuss HB, Johns DC, Kääb S, Tomaselli GF, Kass D, Lawrence JH, and Marbán E.** Reversal of potassium channel deficiency in cells from failing hearts by adenoviral gene transfer: a prototype for gene therapy for disorders of cardiac excitability and contractility. *Gene Ther* 3: 900–912, 1996.
28. **Ter Keurs HEDJ, Rijnsburger WH, van Heuningen R, and Nagelsmit MJ.** Tension development and sarcomere length in rat cardiac trabeculae: evidence of length-dependent activation. *Circ Res* 46: 703–714, 1980.
29. **Von Harsdorf R, Schott RJ, Shen YT, Vatner SF, Mahdavi V, and Nadal-Ginard B.** Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals. *Circ Res* 72: 688–695, 1993.
30. **Weiss DJ, Liggitt D, and Clark JG.** In situ histochemical detection of β-galactosidase activity in lung: assessment of X-gal reagent in distinguishing LacZ gene expression and endogenous β-galactosidase activity. *Hum Gene Ther* 8: 1545–1554, 1997.
31. **Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, and Wilson JM.** Cellular immunity to viral antigenes limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci USA* 91: 4407–4411, 1994.

EXHIBIT I

ELSEVIER

Cardiovascular Research 44 (1999) 294–302

Cardiovascular Research

www.elsevier.com/locate/cardiores
www.elsevier.nl/locate/cardiores

Adenoviral-mediated gene transfer induces sustained pericardial VEGF expression in dogs: effect on myocardial angiogenesis

Daisy F. Lazarous*, Matie Shou, Jonathan A. Stiber, Everett Hodge, Venugopal Thirumurti, Lino Gonçalves, Ellis F. Unger

Experimental Physiology and Pharmacology Section, Cardiology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

Received 22 October 1998; accepted 16 June 1999

Abstract

Objective: Angiogenic peptides like VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) have entered clinical trials for coronary artery disease. Attempts are being made to devise clinically relevant means of delivery and to effect site-specific delivery of these peptides to the cardiac tissue, in order to limit systemic side-effects. We characterized the response of the pericardium to delivery of a replication-deficient adenovirus carrying the cDNA for AdCMV.VEGF$_{165}$, and assessed the effect of pericardial VEGF$_{165}$ on myocardial collateral development in a canine model of progressive coronary occlusion. **Methods:** Ameroid constrictors were placed on the proximal left circumflex coronary artery of mongrel dogs. Ten days later, 6×10^9 pfu AdCMV.VEGF$_{165}$ ($n=9$), AdRSV.β-gal ($n=9$), or saline ($n=7$) were injected through an indwelling pericardial catheter. Transfection efficiency was assessed by X-gal staining. Pericardial and serum VEGF levels were measured serially by ELISA. Maximal myocardial collateral perfusion was quantified with radiolabeled or fluorescent microspheres 28 days after treatment. **Results:** In AdRSV.β-gal-treated dogs, there was extensive β-gal staining in the pericardium and epicardium, with minimal β-gal staining in the mid-myocardium and endocardium. Pericardial delivery of AdCMV.VEGF$_{165}$ resulted in sustained (8–14 day) pericardial transgene expression, with VEGF levels peaking 3 days after infection (>200 ng/ml) and decreasing thereafter. There was no detectable increase in serum VEGF levels. Maximal collateral perfusion, a principal correlate of collateral development and angiogenesis, was equivalent in all groups. **Conclusion:** Adenoviral-mediated gene transfer is capable of inducing sustained VEGF$_{165}$ expression in the pericardium; however, locally targeted pericardial VEGF delivery failed to improve myocardial collateral perfusion in this model.

Keywords: Collateral circulation; Coronary circulation; Gene therapy; Growth factors; Ischemia

1. Introduction

Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) is a secreted, glycosylated, dimeric protein that is structurally related to platelet-derived growth factor (PDGF) [1,2]. VEGF is an endothelial cell-specific mitogen that has been identified as a hypoxia-inducible angiogenic factor [3] and a potential tumor angiogenesis factor in vivo [4]. VEGF has been associated with pathological retinal neovascularization [5,6] and intra-ocular expression of VEGF has been correlated with retinal neovascularization [7]. In experimental models of limb ischemia, VEGF has been demonstrated to promote angiogenesis and collateral development [8–12]. VEGF has also shown promise for myocardial angiogenesis [13–15]; however, parenteral administration of VEGF has been associated with dose-limiting hypotension [15,16] and acceleration of injury-

*Corresponding author. Present address: The Johns Hopkins University School of Medicine, Division of Cardiology, A1 East, Johns Hopkins Bayview Medical Center, 4940 Eastern Avenue, Baltimore, MD 21224, USA. Tel.: +1-410-550-7035; fax: +1-410-550-1183.
E-mail address: dlazarou@welch.jhu.edu (D.F. Lazarous)

Time for primary review 32 days.

0008-6363/99/$ – see front matter
PII: S0008-6363(99)00203-5

induced arterial neointimal accumulation [16], suggesting that local targeting of the growth factor may be essential for clinical use.

Adenoviral-mediated gene transfer has been used successfully to transfer genes to mammalian tissues, and preliminary studies have demonstrated the feasibility of efficient gene transfer to the pericardial sac. [17]. In an attempt to target VEGF protein delivery to cardiac tissues and to limit systemic side-effects, we employed a second-generation adenovirus for transfer of the gene encoding human VEGF to cardiac cells in vivo. An E1a-deleted replication-deficient recombinant adenoviral vector was utilized in which the gene encoding human $VEGF_{165}$ was under the control of the immediate early cytomegalovirus promoter. We exploited the sequestered environment of the pericardial space for site-specific gene transfer, a strategy that might preclude unwanted viral dissemination and enable the vector to evade immune surveillance, thereby prolonging transgene expression. The immediate proximity of the epicardial vasculature and developing collateral circulation to the pericardial space and pericardial fluid suggested that this mode of VEGF delivery might lead to enhancement of myocardial angiogenesis while limiting systemic effects.

Using a β-gal marker gene, our first goal was to determine the anatomic distribution of transgene expression after intra-pericardial virus administration. Second, we sought to characterize the magnitude and time course of $VEGF_{165}$ transgene expression and to determine the distribution of adenovirus in body fluids. Our third goal was to determine whether intrapericardial VEGF gene transfer was capable of improving myocardial collateral perfusion in a well-characterized canine model of progressive coronary occlusion.

2. Methods

The experimental protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* [18] and NIH issuance 3040-2: *Animal Care and Use in the Intramural Program.*

2.1. Surgical instrumentation

Thirty-two mongrel dogs of either sex were obtained from Haycock Kennels, Quakertown, PA. Dogs were anesthetized with acepromazine, 0.2 mg/kg i.m., thiopental sodium, 15 mg/kg i.v., and inhaled methoxyflurane. A left thoracotomy was performed using sterile technique, as previously described [13,16]. An ameroid constrictor (Research Instruments and Manufacturing, Corvallis, OR) was fitted on the proximal left circumflex coronary artery (LCx) before the origin of the first marginal branch. A silastic catheter was positioned in the left atrial appendage for microsphere injections to assess collateral perfusion. Multiple side holes were fashioned in the distal 2 cm of a 6.6-Fr silastic end hole catheter (Bard Access Systems, Salt Lake City, UT), and the catheter was secured in the pericardial space. The pericardium was carefully approximated, and the chest was closed in layers. The termini of the two catheters were positioned in the subcutaneous tissue of the back, the skin was closed, and the dogs were allowed to recover.

2.2. Integrity of the pericardium

Ten days after surgery, the pericardium and catheter were tested for leakage in a pilot dog by injecting 2 ml of contrast agent (Hypaque) into the pericardial space under fluoroscopic guidance. The dye flowed freely in the pericardial space, and no leak was visualized.

2.3. Transduction efficiency of pericardial gene transfer

In one dog, 6×10^9 pfu AdRSV.β-gal was instilled into the pericardial cavity. This gene encodes nuclear-targeted bacterial β-galactosidase. Myocardium, pericardium, lung, intestine, kidney, gonad, brain, liver, and spleen were harvested 72 h after transfection, and bacterial β-galactosidase activity was assessed by 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) staining. The tissue was embedded in paraffin and 5-μm sections were counterstained with Nucleofast red for microscopic analysis.

2.4. Gene expression: serum and pericardial VEGF levels

To establish the ability of transduced tissue to secrete VEGF and to assess the time course of protein secretion, recombinant VEGF protein production was quantified by ELISA in six animals (three VEGF-treated, three controls). Samples of pericardial fluid (withdrawn from the indwelling catheter) and serum samples (forelimb venous puncture) were collected serially: before treatment, and on post-treatment days 1, 3, 5, 7, 10, 14, and 21. Samples were centrifuged at 4°C for 10 min; the supernatant and serum were assayed for human VEGF using a solid-phase ELISA kit according to the manufacturer's instructions (catalog no. DVE00, R&D Systems, Inc., Minneapolis, MN).

2.5. In vitro techniques: biological activity of the secreted VEGF

In order to confirm that the expressed human VEGF was biologically active in canine tissue, we tested the mitogenic activity of the expressed human VEGF on canine endothelial cells in vitro. Endothelial cells were harvested from the saphenous veins of dogs and expanded

in culture. The endothelial cells were infected with AdCMV.VEGF$_{165}$ or AdRSV.β-gal at a multiplicity of infection (moi) of 100 and the supernatant was collected 3 days later. The protein product was quantified in the supernatant of both AdCMV.VEGF$_{165}$ and AdRSV.β-gal-infected cells with a solid-phase ELISA kit as above. Two milliliters conditioned media was obtained from 1×10^6 cells infected 3 days earlier. This was placed on canine endothelial cells previously growth-arrested in 0.5% serum. Absolute cell numbers from triplicate cultures were determined 3 days after exposure to the conditioned media.

2.6. Quantification of E1a-deleted adenovirus in body fluids

Viral titer was quantified in urine, feces, and pericardial aspirates to validate confinement of the adenovirus to the pericardial space. Serial samples were collected from an animal pretreatment and 1, 2, 3, and 6 days after pericardial instillation of 6×10^9 pfu AdCMV.VEGF$_{165}$. Detection and quantification of adenovirus in the samples was done using 293 cells ($TCID_{50}$ assay, i.e. tissue culture infectious dose 50%). In the presence of E1a-deleted adenovirus, 293 cells (embryonic human kidney cells transformed by stable adenovirus E1a gene insertion) exhibit typical adenoviral cytopathic effects within 72–96 h [19] characterized by clumping, detachment, cell lysis, and intra-nuclear basophilic or amphophilic inclusion bodies. Ad5 CMV-LacZ inoculated into normal canine urine served as the positive control; the negative control consisted of a ten-fold dilution of normal canine urine.

2.7. Treatment groups

Dogs were randomized to 6×10^9 pfu AdCMV.VEGF$_{165}$, AdRSV.β-gal or saline, delivered through the indwelling pericardial catheter 10 days after ameroid placement. Selection of the dose was based on our pilot experiments and previous studies [20]. Aliquots of stock AdCMV.VEGF$_{165}$ and AdRSV.β-gal (6×) (generously supplied by GenVec, Inc., Rockville, MD), 10^{10} pfu (volume 1 ml) were stored at −80°C until used. The viral solution was diluted in 3 ml serum-free EBM medium (Clonetics, San Diego, CA) immediately prior to administration. Using sterile technique, the viral solution was injected transcutaneously through the infusion port of the pericardial catheter, followed by 2 ml of EBM medium.

2.8. Analysis of organ toxicity

Using aseptic technique, an 18-gauge needle was inserted into the subcutaneous port of the pericardial catheter on a regular basis to quantify and remove, if necessary, accumulated pericardial fluid. If less than 20 ml of pericardial fluid could be withdrawn, it was returned to the pericardial space. Larger effusions were evacuated to prevent pericardial tamponade. Routine hematologic and biochemical studies were performed weekly on all animals.

2.9. Histology of the heart

Cardiac histological analysis was carried out in 16 animals (saline=6, β-gal=4, and VEGF=6). Transmural sections of the heart at the mid ventricular level were embedded in paraffin (3–5-μm sections) and stained with H&E. The sections were reviewed by a veterinary pathologist who was naïve to treatment assignment. The presence of myocardial fibrosis, inflammation, and increased vascularity were noted.

2.10. Hemodynamic measurements and quantification of collateral perfusion

Regional myocardial blood flow was quantified using the reference sample technique 28 days after treatment [21]. All microsphere blood flow studies were performed in the conscious state during maximal coronary vasodilatation. The dogs were lightly sedated with diazepam 1–2 mg/kg administered through the left atrial catheter. Using local lidocaine anesthesia, a 5F catheter (Cordis Corp.) was inserted into the femoral artery for measurement of arterial pressure and withdrawal of arterial reference samples. Arterial pressure and the electrocardiogram were recorded continuously. Maximal coronary vasodilatation was induced by infusing chromonar 8 mg/kg (Hoechst-Roussel Pharmaceuticals) into the left atrial catheter over 30 min as we have done previously [13,16]. After infusion of chromonar, approximately 3×10^6 radiolabeled microspheres (15 μm) were injected into the left atrial catheter. In a subset of animals, perfusion measurements were made using fluorescent microspheres (Interactive Medical Technology) [22]. Microsphere perfusion measurements were performed in duplicate with two labels (^{46}Ce and ^{95}Nb for radiolabeled microspheres; dual-labeled red, orange or violet dyes for fluorescent microspheres), and the results were averaged. The dogs were heparinized (5000 Units i.v.), killed with an overdose of sodium pentobarbital and KCl, and the myocardium was perfusion-fixed with 10% buffered formaldehyde at physiologic pressure. The heart was cut into 7-mm transaxial slices and examined macroscopically for the presence of infarcts or patchy areas of fibrosis. For radiolabeled microsphere analyses, the two central left ventricular slices were divided into 16 wedges and ranked with respect to perfusion. The four wedges with the highest perfusion were selected to represent the normal zone (NZ); the four wedges with the most compromised perfusion were selected to represent the collateral zone (CZ) as we have done previously [13,16]. For fluorescent microsphere analyses, only the central slice was analyzed. Using a dual wavelength detector system, intact microspheres from tissue digests were characterized with respect to dye ratio and counted using a coulter

system. Tissue digestion and fluorescent microsphere analyses were carried out by Interactive Medical Technology using standard techniques.

Mean NZ and CZ perfusion were calculated from the duplicate perfusion measurements, and maximal collateral perfusion was expressed in relative terms as the CZ/NZ perfusion ratio as we have done previously [13,16]. The CZ/NZ ratio was defined prospectively as the primary study endpoint. Data are expressed as mean±S.E.M.

3. Results

Twenty-six of 32 dogs completed the studies. Five dogs died suddenly, 2–15 days after ameroid placement, presumably from sudden coronary occlusion or ameroid-induced coronary artery spasm. Two of five dogs expired before initial gene transfer. Of the three animals that died after gene transfer, one had received AdCMV.VEGF$_{165}$ and two had received AdRSV.β-gal. Autopsies failed to show an obvious cause of death and death was attributed to arrhythmia from sudden coronary occlusion. A sixth dog (AdCMV.VEGF$_{165}$-treated) died 5 days after randomization; autopsy revealed 250 ml of fluid in the pericardial space and death was attributed to cardiac tamponade. There was discordance between the two measurements of myocardial perfusion in one dog, and it was excluded from the analysis prior to breaking the study code. Thus, perfusion measurements are reported for 25 dogs. One additional dog was used for a determination of in vivo β-gal transfection efficiency.

3.1. In vitro VEGF production and biological activity

VEGF was detected in the supernatant of AdCMV.VEGF$_{165}$ cells but not in the cells infected with AdRSV.β-gal. Peak VEGF levels of approximately 1 μg/million cells/day were observed between days 3 and 5 after infection, tapering off thereafter (Fig. 1). The expressed human VEGF induced a two-fold increase in proliferation of canine endothelial cells, indicating that the VEGF was biologically active, and that its activity was conserved between species (data not shown).

3.2. Pericardial transfection efficiency

There was extensive β-gal expression in the parietal pericardium (Fig. 2a) and epicardium (Fig. 2b). Reporter gene transfer was almost entirely restricted to pericardial and epicardial cells, with scattered sparse expression in the mid-myocardium, and no detectable staining of the endocardium.

3.3. Pericardial and serum VEGF levels

Pre-treatment VEGF levels were negligible in both virus-treated groups. Transgene expression was evident 24 h after viral infection, and peak pericardial VEGF levels were recorded on day 3 in AdCMV.VEGF-treated dogs (>200 ng/ml). VEGF levels diminished to ≈15 ng/ml by day 8, and tapered off gradually thereafter, such that the total duration of the response was ≈14 days (Fig. 3). Serum VEGF levels did not increase from pretreatment values. Neither pericardial nor serum VEGF levels were elevated in AdRSV.β-gal treated animals.

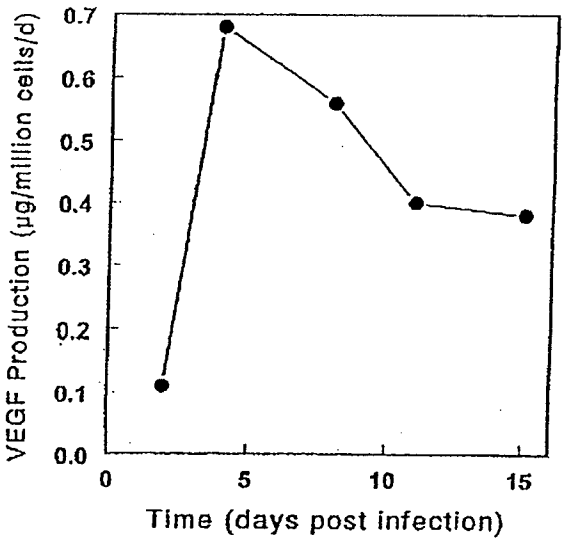


Fig. 1. In vitro VEGF production. VEGF was detected in the supernatant of AdCMV.VEGF$_{165}$ cells but not in the cells infected with AdRSV.β-gal. Peak VEGF levels of approximately 1 μg/million cells/day were produced 3–5 days after infection. The expressed human VEGF induced a two-fold increase in proliferation of canine endothelial cells, indicating that the biological activity was conserved between species.

3.4. Detection of recombinant adenovirus in body fluids

There was no detectable shedding of virus in the fecal or urine samples. At 6 days, the pericardial aspirate remained infectious and contained 5×10^2/ml $TCID_{50}$ virus, still capable of replication in 293 cells.

3.5. Analysis of organ toxicity

All AdCMV.VEGF$_{165}$-treated animals developed significant pericardial effusions, 55–180 ml in volume. One AdCMV.VEGF$_{165}$-treated animal expired from pericardial tamponade with a 250-ml hemorrhagic inflammatory effusion (elevated polymorphonuclear leucocyte count). The aspirated fluid was cultured in all cases and found to be free of bacterial infection. None of the AdRSV.β-gal-treated animals had significant effusions (>20 ml).

3.6. Collateral conductance and CZ/NZ perfusion ratios

Microsphere perfusion determinations, performed in

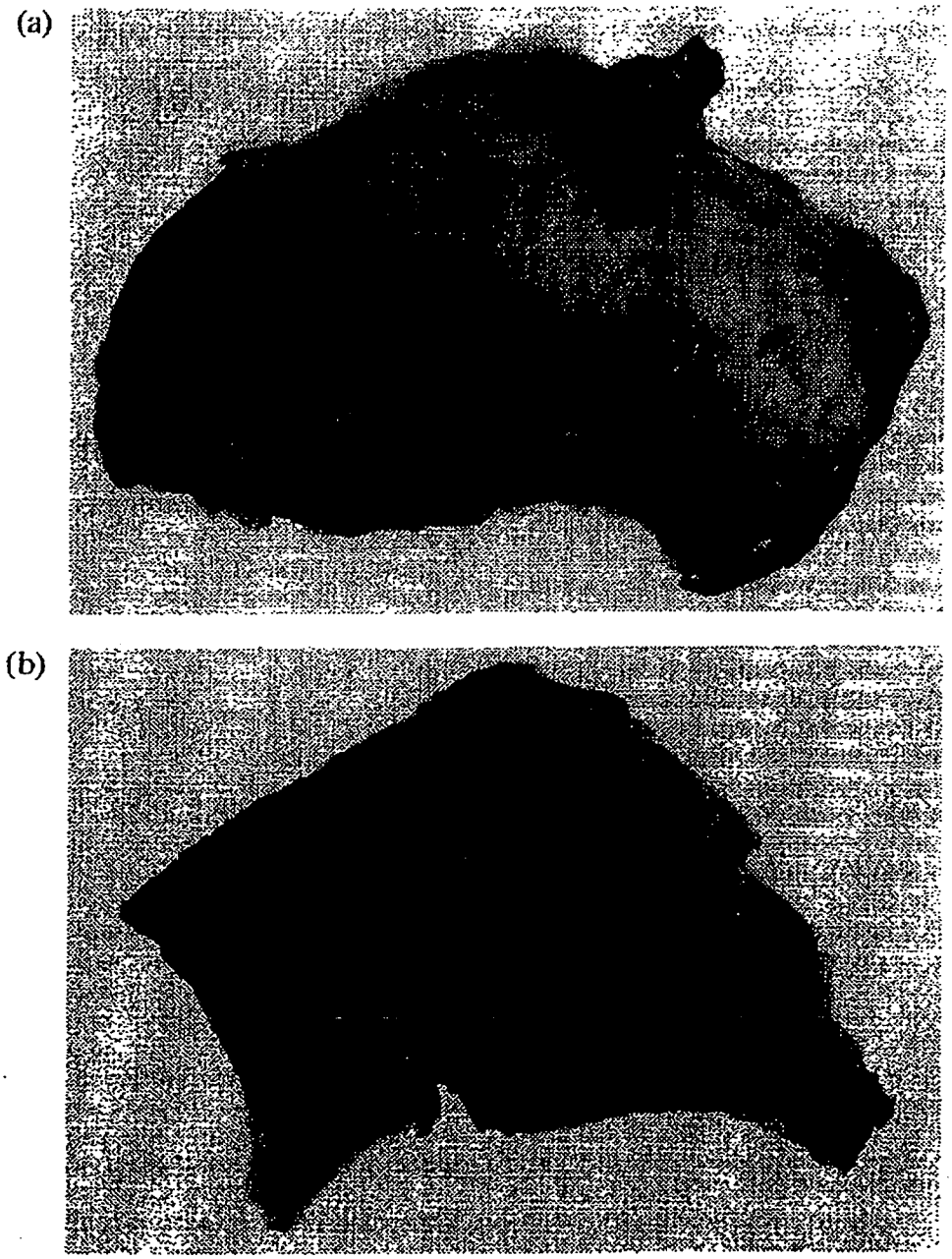


Fig. 2. Expression of β-galactosidase after intrapericardial instillation of 6×10^9 pfu AdRSV.β-gal. The heart was harvested 72 h post-treatment and X-gal histochemical staining was performed. Panel A: reporter gene transfer was almost entirely restricted to the parietal pericardial cells, and panel B: visceral pericardial cells (epicardium). There was scattered scanty expression in the mid-myocardium, and no detectable staining of the endocardium. Distant tissues were free of β-gal staining.

duplicate, showed excellent intra-animal agreement. In the normally-perfused territory of the left anterior descending coronary artery, maximal coronary conductance was similar in the three treatment groups on day 28 (Table 1). Maximal collateral conductance in the territory of the occluded LCx was similar in all groups, and the CZ/NZ ratios were virtually identical in the three treatment groups. For the critical comparison between the AdRSV.β-gal and AdCMV.VEGF$_{165}$ groups, mean CZ/NZ ratios were 0.36±0.03 and 0.36±0.04, respectively.

3.7. Histological analysis

No cytopathic effects were grossly evident in the

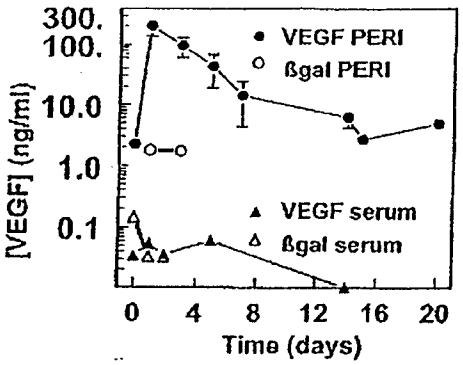


Fig. 3. Line plot of pericardial and serum VEGF levels from six animals (three AdCMV.VEGF-treated and three AdRSV.β-gal-treated). Transgene expression was evident 24 h after viral infection, with the measurable increase in [VEGF] persisting for ≈14 days. Pretreatment VEGF levels were negligible in both virus-treated groups. The VEGF concentration in pericardial fluid peaked on day 3 in AdCMV.VEGF-treated dogs (>200 ng/ml). VEGF levels diminished to ≈15 ng/ml by day 8, and tapered off gradually thereafter. VEGF levels were not elevated in AdRSV.β-gal-treated animals. Serum VEGF concentrations remained at pretreatment levels in all groups.

pericardium or surrounding lungs at the time of tissue harvest, i.e. structures were relatively free of adhesions. X-gal staining of distant organs did not reveal β-gal expression in any tissue.

Histological analysis of the heart revealed no difference between treatment groups in the degree of vascularity, fibrosis or inflammation or thrombosis. Microscopic angiomas or hemangiomas were not noted.

4. Discussion

VEGF is one of several angiogenic heparin-binding growth factors reported to promote collateral development in animal models of myocardial and limb ischemia [8–15]. In previous experiments in a canine model, we showed that VEGF enhanced coronary collateral development when administered repeatedly into collateral-dependent myocardium [13]. VEGF has also been reported to improve coronary collateral function in pigs when infused chronically near the point of coronary occlusion [14]. Enhancement of coronary collateral perfusion has not been attained, however, following relatively brief periods of VEGF treatment [15,16]. Moreover, intracoronary [15] and systemic arterial [16] administration of the growth factor has been associated with severe dose-limiting side effects. VEGF is a potent vascular permeability factor [2] and endothelium-dependent vasodilator [23], and causes severe hypotension when administered parenterally [15,16]. VEGF has also shown the potential to induce neointimal accumulation after vascular injury [16]. Thus, although VEGF appears to have the potential to promote myocardial angiogenesis, specific local targeting and/or extended peptide delivery may be essential to induce myocardial angiogenesis in the absence of toxicity [15,16]. For these reasons, we considered gene transfer as a means to bring about localized myocardial targeting of VEGF, with extended delivery of protein to maximize therapeutic effect.

Arterial gene transfer is currently under investigation as a means to promote therapeutic angiogenesis in ischemic limbs, utilizing a hydrogel polymer-coated balloon to establish direct contact of naked VEGF plasmid cDNA with the arterial wall [24,25]. This technique has important limitations due to the inherent inefficiency of direct arterial plasmid gene transfer, a problem that is magnified in atherosclerotic arteries. Moreover, in at least one investigation in which this technique has been successful, a generalized drug effect was observed, [26] suggesting that limited, locally targeted therapy may not be attainable with this method.

Table 1
Myocardial perfusion and the CZ/NZ perfusion ratio*

	Control			AdRSV.β-gal			AdCMV.VEGF$_{165}$		
	Perfusion (ml/min/g)		CZ/NZ ratio	Perfusion (ml/min/g)		CZ/NZ ratio	Perfusion (ml/min/g)		CZ/NZ ratio
	CZ	NZ		CZ	NZ		CZ	NZ	
	1.71	6.37	0.27	2.57	6.94	0.37	2.86	7.63	0.38
	1.93	6.90	0.28	2.98	6.61	0.45	3.93	7.38	0.53
	2.53	5.99	0.42	2.53	4.84	0.52	1.98	3.93	0.50
	1.92	5.51	0.35	1.5	5.32	0.28	1.29	4.32	0.30
	1.49	3.59	0.42	2.28	6.05	0.37	4.26	13.36	0.32
	2.51	5.67	0.44	1.51	4.03	0.38	0.74	4.80	0.16
	1.52	3.89	0.39	1.79	5.06	0.35	1.84	4.54	0.40
				0.96	6.18	0.15	2.47	8.53	0.29
				1.67	4.28	0.39	2.13	5.34	0.39
Mean	1.95	5.42	0.37	1.98	5.48	0.36	2.39	6.65	0.36
S.E.M.	0.16	0.47	0.03	0.22	0.34	0.03	0.38	1.00	0.04

*Collateral zone (CZ) and normal zone (NZ) myocardial perfusion and the CZ/NZ ratio in control dogs, AdRSV.β-gal-treated dogs, and AdCMV.VEGF$_{165}$-treated dogs. Values are mean±S.E.M. Differences between groups are not significant.

Several investigators have demonstrated that second generation adenoviral vectors function efficiently as gene delivery vehicles in vivo [27,28], superior in efficiency to plasmid DNA injection [29]. Successful intramyocardial injection of adenoviral vectors has been demonstrated in mammals [20,32]. Recently, intracoronary injection of a recombinant adenovirus expressing human FGF-5 was shown to increase perfusion and contractile function in the ischemic porcine heart [30]. Intracoronary gene delivery appears promising, although the potential for viral dissemination to non-target tissues poses an important concern. Viral receptors are found on the surface of a variety of cell types, and generalized dissemination of angiogenic genes could lead to undesirable neovascularization in sites such as the retina or occult tumors. Host immune responses to foreign adenoviral antigens pose an additional barrier for gene therapy, limiting the duration of gene expression [31].

We evaluated pericardial VEGF gene transfer as a means to target the myocardium and avoid the potential problems associated with systemic adenoviral dissemination. E1a-deleted replication-deficient adenovirus with the gene encoding $VEGF_{165}$ was delivered to the pericardial space. We hypothesized that the pericardium might provide an abundant surface area for gene transfer as well as an inherent means for viral containment. Furthermore, the limited pericardial volume of distribution would tend to favor gene transfer by maximizing the concentration of the virus, and would serve to maintain the concentration of the expressed VEGF gene product at a relatively high level. In essence, the pericardium might function as a paracrine organ, producing high localized VEGF levels in direct apposition to the epicardial arteries and developing collaterals, leading to myocardial angiogenesis.

The pericardial space has been evaluated previously as a reservoir for angiogenic growth factor delivery [32,33]. Pericardial injection of basic fibroblast growth factor has been reported to enhance myocardial angiogenesis [36] and limit infarct size after coronary embolization [37]. Woody et al. have reported gene transfer to the pericardium [17]. Thus, these studies, taken together, suggest that pericardial gene transfer might provide an effective means to induce myocardial angiogenesis.

In the present study, we demonstrated that gene transfer to non-replicating pericardial and epicardial cells using recombinant adenovirus vectors resulted in acceptable gene targeting efficiency and expression of the VEGF transgene over a 10–14-day period. Although this strategy was highly effective in directing pericardial VEGF expression, the pericardial VEGF so produced was ineffective in promoting collateral development. There are a number of potential explanations for the apparent lack of effect.

4.1. Adequacy of VEGF production

It is worthwhile to contrast the calculated mass of VEGF produced by gene transfer in the present experiment to the quantities of the VEGF peptide administered in previous studies in which it was found to be effective. We observed peak steady state VEGF levels of approximately 200 ng/ml in a pericardial fluid volume of roughly 200 ml: an estimated mass of approximately 40 μg. Considering that the pericardial VEGF concentration decreased to 15 ng/ml by day 8, the total quantity of VEGF produced in the present investigation, integrated over the 2-week period of expression, was probably an order of magnitude less than the cumulative injected dose we previously found to be effective (45 μg/day×20 doses=900 μg) [13]. On the other hand, the quantity of VEGF produced in this experiment significantly exceeded the dose used by Harada et al. (0.06 μg/day×28 days=1.68 μg) [14].

4.2. Direction of VEGF delivery across the vascular wall

The presence of VEGF in the pericardial space presents the growth factor to the abluminal (rather than the luminal) side of the vasculature. Though this unconventional mode of VEGF administration might be responsible for its lack of effect in this experiment, Harada et al. [14] found perivascular infusion of VEGF (mixed with heparin) to be effective, a method that also delivered VEGF to the abluminal aspect of the vasculature. Two key differences between the present and previous investigations should be underscored: Harada et al. [14] used a longer duration of infusion (4 weeks, although VEGF potency was reduced by a factor of 2.5 at the end of their study), and VEGF and heparin were co-administered in their study. VEGF is a heparin-binding growth factor, and the interactions between VEGF and heparin have not been well-characterized in vivo [34,35].

4.3. Preservation of VEGF activity across species

We considered the possibility that human $VEGF_{165}$ was not active in canine cells. In the present study, the expressed human $VEGF_{165}$ induced proliferation of canine endothelial cells in vitro. Moreover, we previously found human $VEGF_{165}$ to be effective on canine endothelial cells in vitro [13].

4.4. Role of inflammation

It is plausible that adenoviral-related inflammation had a negative influence on collateral development in this study. We found, however, that collateral function was not depressed in AdRSV.β-gal-treated animals (relative to saline-treated controls), suggesting that this was not the case. Animals treated with the β-gal virus did not develop hemodynamically significant pericardial effusions, but large effusions were encountered consistently in AdCMV.VEGF-treated dogs, requiring drainage to avert hemodynamic compromise. Leucocyte counts were gener-

ally elevated in these aspirates, indicative of an inflammatory response. Thus, it appears that a specific biological effect of VEGF was responsible for the sizable and hemodynamically significant effusions characteristic of these dogs. VEGF-induced vascular hyperpermeability may have played an important role in this regard [2]. The requirement to tap the effusions subsided by day 15 post-transfection, coinciding with waning VEGF expression.

Recently it has been reported that the plasmid for VEGF injected in the border zone of myocardial infarct in rats created angiomas that did not contribute to regional myocardial blood flow [36]. VEGF gene delivery to non-ischemic muscle in mice led to an increase in vascular channels and hemangiomas, associated with local high serum VEGF levels [37]. Thus, there is concern that over expression/high doses of growth factors may have deleterious effects on both ischemic and non-ischemic tissue. We did not observe angiomas or hemangiomas in this study.

In summary, adenoviral-mediated gene transfer is capable of inducing sustained $VEGF_{165}$ expression in the pericardium. Pericardial VEGF levels in the 200 ng/ml range were achieved in the absence of an increase in systemic VEGF levels. Despite appreciable local VEGF production, however, myocardial collateral perfusion was not improved in this model. These data add to a growing number of studies on the effects of VEGF on myocardial angiogenesis – a biological effect of VEGF has been demonstrated in some but not all of these investigations. Additional studies are needed to better characterize the response of the coronary collateral circulation to VEGF. The optimal VEGF dose, the timing, duration, and route of VEGF delivery, and the potential role of heparin co-administration are important issues that merit further study. Moreover, it remains to be determined whether pericardial gene transfer of alternative forms of VEGF or other angiogenic growth factors might foster collateral growth.

Acknowledgements

The authors thank V. Hampshire, J. Bacher, and their staffs for providing veterinary care, GenVec, Inc. for supplying the recombinant adenoviruses, and Sanjay Shah for technical assistance.

References

[1] Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989;246:1306–1309.

[2] Keck PJ, Hauser SD, Krivi G et al. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989;246:1309–1312.

[3] Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992;359:843–845.

[4] Plate K, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. Nature 1992;359:845–848.

[5] Aiello LP, Pierce EA, Foley ED et al. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 1995;92:10457–10461.

[6] Stone J, Itin A, Alon T et al. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. J Neurosci 1995;15:4738–4747.

[7] Pe'er J, Shweiki D, Itin A et al. Hypoxia-induced expression of vascular endothelial growth factor by retinal cells is a common factor in neovascularizing ocular diseases. Lab Invest 1995;72:638–645.

[8] Banters C, Asahara T, Zheng LP et al. Physiological assessment of augmented vascularity induced by VEGF in ischemic rabbit hindlimb. Am J Physiol 1994;267:H1263–1271.

[9] Takeshita S, Zheng LP, Brogi E et al. Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. J Clin Invest 1994;93:662–670.

[10] Takeshita S, Pu LQ, Stein LA et al. Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. Circulation 1994;90:II228–II234.

[11] Asahara T, Banters C, Zheng LP et al. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation 1995;92:II365–II371.

[12] Banters C, Asahara T, Zheng LP et al. Site-specific therapeutic angiogenesis after systemic administration of vascular endothelial growth factor. J Vasc Surg 1995;21:314–324.

[13] Banai S, Jaklitsch MT, Shou M et al. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. Circulation 1994;89:2183–2189.

[14] Harada K, Friedman M, Lopez JJ et al. Vascular endothelial growth factor administration in chronic myocardial ischemia. Am J Physiol 1996;270:H1791–H1802.

[15] Hariawala MD, Horowitz JJ, Esakof D et al. VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. J Surg Res 1996;63:77–82.

[16] Lazarous DF, Shou M, Scheinowitz M et al. Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury. Circulation 1996;94:1074–1082.

[17] Woody M, Mehdi K, Zipes DP et al. High efficiency adenovirus-mediated pericardial gene transfer in vivo [abstract]. J Am Coll Cardiol 1996;27(Suppl A):31A.

[18] National Research Council. Guide for the care and use of laboratory animals, Washington, DC: National Academy Press, 1996.

[19] Dulbecco E, Ginsberg A, editors, Virology, New York: Harper and Row, 1980, p. 881.

[20] Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circ Res 1993;73:1202–1207.

[21] Heymann MA, Payne BD, Hoffman JIE, Rudolph AM. Blood flow measurements with radionuclide-labelled particles. Prog Cardiovasc Dis 1977;20:55–79.

[22] Austin GE, Tuvlin MB, Martino-Saltzman D, Hunter R, Justicz AG. Determination of regional myocardial blood flow using fluorescent microspheres. Am J Cardiovasc Pathol 1993;4:352–357.

[23] Ku DD, Zaleski JK, Liu S, Brock TA. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. Am J Physiol 1993;265:H586–H592.

[24] Isner JM, Walsh K, Symes J et al. Arterial gene therapy for therapeutic angiogenesis in patients with peripheral artery disease [news]. Circulation 1995;91:2687–2692.

[25] Isner JM, Pieczek A, Schainfeld R et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996;348:370–374.

[26] Asahara T, Chen D, Kearney M et al. Accelerated re-endothelialization and reduced neointimal thickening following catheter transfer of phVEGF$_{165}$ [abstract]. J Am Coll Cardiol 1996;27(Suppl A):1A.

[27] Rade JJ, Schulick AH, Virmani R, Dichek DA. Local adenoviral-mediated expression of recombinant hirudin reduces neointima formation after arterial injury. Nat Med 1996;2:293–298.

[28] Muhlhauser J, Merrill MJ, Pili R et al. VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis in vivo. Circ Res 1995;77:1077–1086.

[29] French BA, Mazur W, Geske RS, Bolli R. Direct in vivo gene transfer into porcine myocardium using replication-deficient adenoviral vectors. Circulation 1994;90:2414–2424.

[30] Giordano FJ, Ping P, McKirnan MD et al. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. Nat Med 1996;2:534–539.

[31] Gilgenkrantz H, Duboc D, Juillard V et al. Transient expression of genes transferred in vivo into heart using first-generation adenoviral vectors: role of the immune response. Hum Gene Ther 1995;6:1265–1274.

[32] Landau C, Jacobs AK, Haudenschild CC. Intrapericardial basic fibroblast growth factor induces myocardial angiogenesis in a rabbit model of chronic ischemia. Am Heart J 1995;129:924–931.

[33] Uchida Y, Yanagisawa-Miwa A, Nakamura F et al. Angiogenic therapy of acute myocardial infarction by intrapericardial injection of basic fibroblast growth factor and heparin sulfate: An experimental study. Am Heart J 1995;130:1182–1188.

[34] Cohen T, Gitay-Goren H, Sharon R. VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells. J Biol Chem 1995;270:11322–11326.

[35] Terman B, Khandke L, Dougher-Vermazan M et al. VEGF receptor subtypes KDR and FLT1 show different sensitivities to heparin and placenta growth factor. Growth Factors 1994;11:187–195.

[36] Schwarz ER, Spealman MT, Patterson M et al. Effect of intra myocardial injection of DNA expressing vascular endothelial growth factor in myocardial infarct tissue in the rat heart: angiogenesis and angioma formation. Circulation 1998;456(Suppl 1):2397.

[37] Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. Mol Cell 1998;2:549–558.

EXHIBIT J

Targeted Expression of a Dominant-Negative $K_v4.2$ K^+ Channel Subunit in the Mouse Heart

Alan D. Wickenden, Paul Lee, Rajan Sah, Qian Huang, Glenn I. Fishman, Peter H. Backx

Abstract—Action potential duration is prolonged in many forms of heart disease, often as a result of reductions in Ca^{2+}-independent transient outward K^+ currents (ie, I_{to}). To examine the effects of a primary reduction in I_{to} current in the heart, transgenic mice were generated that express a dominant-negative N-terminal fragment of the $K_v4.2$ pore-forming potassium channel subunit under the control of the mouse α-myosin heavy chain promoter. Two of 6 founders died suddenly, and only 1 mouse successfully transmitted the transgene in mendelian fashion. Electrophysiological analysis at 2 to 4 weeks of age demonstrated that I_{to} density was specifically reduced and action potential durations were prolonged in a subset of transgenic myocytes. The heterogeneous reduction in I_{to} was accompanied by significant prolongation of monophasic action potentials. In vivo hemodynamic studies at this age revealed significant elevations in the mean arterial pressure, peak systolic ventricular pressures, and $\pm dP/dt$, indicative of enhanced contractility. Surprisingly, by 10 to 12 weeks of age, transgenic mice developed clinical and hemodynamic evidence of congestive heart failure. Failing transgenic hearts displayed molecular and cellular remodeling, with evidence of hypertrophy, chamber dilatation, and interstitial fibrosis, and individual myocytes showed sharp reductions in I_{to} and I_{K1} densities, action potential duration prolongation, and increased cell capacitance. Our results confirm that $K_v4.2$ subunits contribute to I_{to} in the mouse and demonstrate that manipulation of cardiac excitability may secondarily influence contractile performance. (*Circ Res.* 1999;85:1067-1076.)

Key Words: K^+ channel ■ transgenic ■ cardiac electrophysiology ■ mouse ■ heart failure

Heart disease results from a variety of primary genetic and acquired stimuli.[1–6] Regardless of the initiating cause, neurohumoral and cellular responses typically cause hypertrophic remodeling of the heart characterized by genetic, biochemical, structural, and functional alterations within myocytes.[7–10] One commonly observed feature of diseased myocardium is action potential prolongation.[11–13] Although the cardiac action potential arises from the composite activity of numerous ion channels and transporters, its prolongation correlates strongly with reductions in repolarizing K^+ currents and K^+ channel gene expression, particularly the Ca^{2+}-independent transient outward current (I_{to}).[11,13–15]

Heterogeneous action potential prolongation is associated with an enhanced propensity for cardiac arrhythmias.[12,13,16,17] The abnormally prolonged repolarization predisposes to early and delayed afterdepolarizations and triggered activity, whereas the dispersion of refractoriness may facilitate stable reentry.[16–18] This association between action potential prolongation as a result of reduced K^+ currents and arrhythmias is highlighted by the high incidence of arrhythmogenesis in acquired and inherited forms of the long-QT syndrome.[19,20]

Action potential prolongation also strongly influences $[Ca^{2+}]_i$ transient magnitude.[21] This effect appears to underlie the positive inotropic actions of α-adrenergic receptor activation[22,23] that occurs both acutely and chronically in heart disease.[10] In turn, elevated Ca^{2+} may contribute to cellular hypertrophy commonly seen in heart disease by acting as a stimulus for cellular growth through the activation of a number of cell signaling pathways,[9] such as the recently described calcineurin-dependent pathway.[24]

In this study, we examined the phenotypic consequences of a primary reduction in repolarizing transient outward currents in the heart. We created transgenic mice overexpressing an N-terminal fragment of $K_v4.2$, a strategy predicted to specifically reduce I_{to}[25–29] by a dominant-negative mechanism.[30] We found that expression of the transgene was poorly tolerated; of 6 transgenic founders, 2 died suddenly, 3 others failed to achieve germline transmission, and only 1 line transmitted the transgene in mendelian fashion. In this surviving line, neonatal hearts appeared structurally normal but displayed increased contractility in vivo, and myocytes showed specific although heterogeneous reduction in I_{to}

Received August 30, 1999; accepted September 10, 1999.

From the Department of Medicine, Division of Cardiology (P.H.B., A.D.W.), The Toronto Hospital, and the Departments of Medicine and Physiology (P.H.B., A.D.W., R.S.), University of Toronto, Ontario, Canada, and the Section of Myocardial Biology, Cardiovascular Institute, Departments of Medicine (P.L., G.I.F.), Physiology & Biophysics and Biochemistry and Molecular Biology (G.I.F.), Mount Sinai School of Medicine, New York, NY. A.D.W.'s current address is ICAgen Inc, Durham, NC. Q.H.'s current address is Merck & Co, Inc, West Point, Pa.

Correspondence to Glenn I. Fishman, MD, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1269, New York, NY 10029. E-mail fishmg01@doc.mssm.edu

Circulation Research is available at http://www.circresaha.org

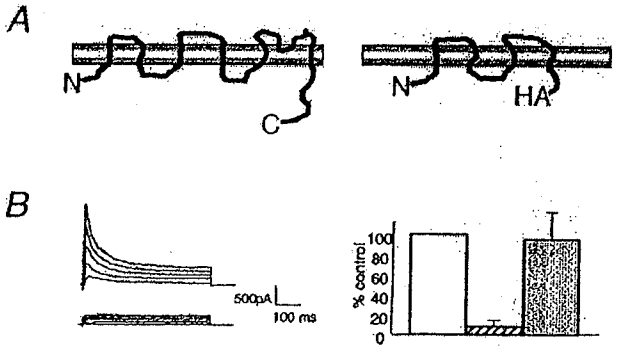


Figure 1. K^+ channel constructs and in vitro characterization. A, Schematic diagram of the $K_v4.2$ protein, which contains 6 transmembrane segments (S1–S6) and a pore-forming loop between S5 and S6. A truncated N-terminal fragment of $K_v4.2$ ($K_v4.2N$) encoding the first 311 amino acids (S1–S4) of the parent molecule was generated. The truncated N-terminal construct contains those sequences that are known to be important for subtype-specific tetramerization but lacks the pore-forming P-loop. An HA epitope tag was placed in frame at the C terminus of the $K_v4.2N$ gene. B, Whole-cell voltage-clamp records show typical families of outward currents recorded in tsa201 cells transfected with full-length $K_v4.2$ plus a 3-fold excess of vector alone (left, top) or with full-length $K_v4.2$ plus a 3-fold excess of $K_v4.2N$-HA (left, bottom). $K_v4.2$ induced the expression of large, inactivating outward currents that were absent in cells cotransfected with a 3-fold excess of $K_v4.2N$-HA. Right, Relative current densities in control cells ($K_v4.2$ alone, open bar), in cells cotransfected with a 3-fold excess of $K_v4.2N$-HA (solid bar), and in cells cotransfected with a 3-fold excess of a truncated $K_v1.4$ fragment ($K_v1.4N$, hatched bar). Data are mean±SEM of 5 to 27 experiments.

currents. By 10 to 16 weeks of age, transgenic mice developed progressive cardiac hypertrophy and chamber dilatation, culminating in congestive heart failure and death.

Materials and Methods

Plasmid Constructs

Expression plasmids encoding rat $K_v4.2$, $K_v1.4$, and $K_v2.1$ were kindly provided by Drs J. Nerbonne (Washington University, St. Louis, Mo), M.M. Tamkun (Colorado State University, Fort Collins, Colo), and R. Joho (University of Texas, Dallas, Tex), respectively. The dominant-negative $K_v4.2N$-HA construct was prepared by fusing amino acids 1 to 311 of wild-type $K_v4.2$ in frame to an HA epitope tag (Figure 1A).

Generation of Kv4.2N Transgenic Mice

The $K_v4.2N$-HA gene was targeted to the heart using regulatory elements from the mouse α-myosin heavy chain gene (αMHC; clone 26, kindly provided by Dr J. Robbins, University of Cincinnati, OH). Transgenic mice were generated in the B6XCBAF2 background as previously described.[31] Mice expressing the transgene were initially identified by Southern blotting and subsequently by PCR.

Northern and Western Blot Analyses

Northern blot analyses was performed as described previously using probes to phospholamban (PLB), sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), atrial natriuretic factor (ANF), β-myosin heavy chain (βMHC), $K_v4.2$, and GAPDH.[32] Crude membrane preparations from pooled mouse hearts were analyzed by Western blot as previously described,[28] using a monoclonal antibody to the HA epitope tag (Boehringer Mannheim) and rabbit polyclonal antisera raised against an amino-terminal epitope of the rat $K_v4.2$ protein.

Isolation of Mouse Ventricular Myocytes

Mouse hearts were isolated and perfused in retrograde fashion with a solution of collagenase and protease using a slight modification of a procedure previously described.[28]

Electrical Recordings in Ventricular Myocytes, Oocytes, and tsa201 Cells

Two-electrode voltage-clamp recordings of *Xenopus laevis* oocytes were performed as previously described.[33] Membrane currents were recorded from mammalian tsa201 and from Ca^{2+}-tolerant, rod-shaped ventricular myocytes using the whole-cell configuration of the patch-clamp technique,[34] essentially as previously described.[35]

I_{to} was measured as peak current elicited by the depolarizing voltage step minus the current remaining at the end of the 500-ms voltage step (ie, I_{500}). I_{K1} was measured as a Ba^{2+}-sensitive current using 500-ms steps from −130 to 0 mV (10-mV increments) from the holding potential in the presence and absence of 0.3 mmol/L $BaCl_2$. Action potentials were elicited at a frequency of 1 Hz and were recorded in the absence of Cd^{2+}.

Microsurgical Methods and In Vivo Hemodynamic Measurements

Mice were anesthetized, and the carotid artery was cannulated with polyethylene tubing (PE-200), which was connected to a TXD-310 low-compliance pressure transducer (MicroMed) and amplified by a blood pressure analyzer (BPA model 300, MicroMed). Heart rate, aortic pressure, left ventricular (LV) systolic pressure, LV diastolic pressure, and the maximum and minimum first derivatives of the LV pressure ($+dP/dt_{max}$ and $-dP/dt_{max}$, respectively) were recorded.

Echocardiographic Assessment

Mice were anesthetized and examined by transthoracic echocardiography using a 12-MHz probe (Hewlett Packard). Ejection velocity, end-systolic (ESD), and end-diastolic (EDD) dimensions were recorded and fractional shortening (FS) was calculated as: FS=(EDD–ESD)/EDD.

Monophasic Action Potentials

Hearts were retrogradely perfused with Tyrode's solution at 37°C, and action potentials were recorded from the surface of the left ventricles using a close-bipolar configuration.[36] The times for 50% (APD_{50}) and 90% (APD_{90}) repolarization were recorded.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Characterization of the Kv4.2N Dominant-Negative Construct in *Xenopus* Oocytes

Initial experiments were designed to test the efficacy and specificity of the epitope-tagged dominant-negative $K_v4.2N$ construct, schematized in Figure 1A. Coinjection of the HA-tagged truncated $K_v4.2N$-HA with the full-length $K_v4.2$ into *Xenopus* oocytes caused a significant ($P<0.01$), dose-dependent reduction in current (ie, peak current amplitude was 1.13±0.13 μA [n=16], 0.25±0.04 μA [n=12], and −0.18±0.11 μA [n=7] when the ratio of $K_v4.2N$ to $K_v4.2$ was 0:1, 1:1, and 3:1). In contrast, peak current was not affected by coinjection of a 3-fold excess of the parental pGW1H plasmid with $K_v4.2$ (1.11±0.09 μA)[23] or coinjection of a 3- to 10-fold excess of $K_v4.2N$-HA with $K_v1.4$ or $K_v2.1$ (data not shown). Similar dominant-negative inhibition was observed in tsa201 mammalian cells. Figure 1B shows typical families of outward currents recorded in cells transfected either with $K_v4.2$ plus a 3-fold excess of vector alone (top) or with $K_v4.2$ plus a 3-fold excess of $K_v4.2N$-HA (bottom).

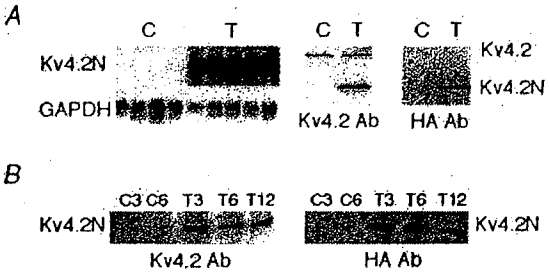


Figure 2. A, Northern (left) and Western (right) blots detecting K_v4.2N-HA transgene expression in hearts from control (C) and transgenic (T) mice at 3 weeks of age. Using antibodies against the N terminus of the K_v4.2 channel, the truncated K_v4.2N is only expressed in transgenic mice, whereas full-length K_v4.2 gene is readily detected in both control and transgenic mouse hearts. Note that in transgenic mice, expression of K_v4.2N-HA is much higher (ie, ≈10-fold) than the endogenous full-length K_v4.2 protein. B, Time course of K_v4.2N expression measured by Western blotting of mouse hearts with antibodies raised against the N-terminal fragment (ie, K_v4.2 Ab) or the HA epitope (ie, HA Ab). No K_v4.2N is detected in hearts from controls at 3 (C3) and 6 (C6) weeks of age. Levels of expression are similar in transgenic mice at 3 (T3), 6 (T6), and 12 (T12) weeks of age.

K_v4.2N-HA coexpression significantly ($P<0.001$) reduced current densities from 375±84 pA/pF (n=17) to only 7.9±6.4% of control (n=6). In contrast, a 3-fold excess of K_v4.2N-HA did not affect K_v1.4 current densities. Collectively, these results establish that the K_v4.2N-HA fragment potently and specifically inhibits K_v4.2-based currents, as expected from previous results.[37]

Generation and Initial Characterization of Transgenic Mice

Six founders harboring the K_v4.2N transgene were initially identified from a total of 34 live births screened. Two of these founders died suddenly before yielding any progeny and were discarded without further analysis. Three additional founders produced multiple litters with no transgenic offspring. The sixth founder yielded several litters with a normal distribution of transgenic progeny from which a line was established and then died suddenly as well. These initial observations of the F_0 transgenic mice strongly suggested that expression of the dominant-negative K^+ channel subunit in the heart was deleterious.

Transgene expression in the hearts of mice from the established line was examined at both the transcript and protein levels, as illustrated in Figure 2A. Northern blot analysis of cardiac RNA revealed abundant accumulation of the MHCα-K_v4.2N transcript (left). Western blot analysis using an antibody directed against an amino-terminal epitope of K_v4.2 (K_v4.2 Ab) detected expression of the endogenous K_v4.2 protein in both control and transgenic hearts, whereas the truncated HA-tagged protein was expressed exclusively in transgenic hearts (right). The identity of the truncated K_v4.2N protein was confirmed by immunoblotting with a monoclonal antibody directed against the HA epitope tag. The level of K_v4.2N expression remained unchanged from 3 weeks to 12 weeks of age (Figure 2B).

Clinical Course

Although transgenic mice initially appeared healthy and indistinguishable from nontransgenic littermates, by 12 to 13 weeks of age most transgenic mice developed obvious signs of congestion consistent with biventricular heart failure. Mice at this stage were dyspneic and sedentary and frequently appeared edematous, as illustrated in Figure 3A. Hearts isolated from transgenic mice at 13 weeks were enlarged (Figure 3B and 3C), and heart weight/body weight ratios were significantly increased compared with littermate controls, as summarized in Table 1. On cross section, ventricular hypertrophy and chamber dilatation, especially in the left atrium, was observed (Figure 3D through 3F). There was also evidence of myocyte hypertrophy, myocyte cell loss, and interstitial fibrosis and cellularity (Figure 3G through 3I). A markedly dilated left atrium with organized thrombus forma-

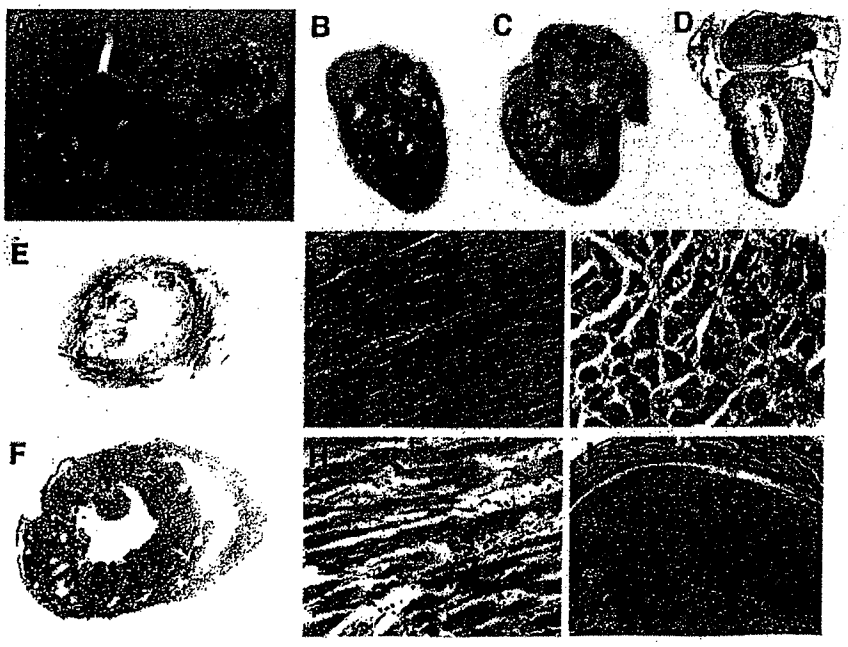


Figure 3. Cardiomyopathy in K_v4.2N transgenic mice. A, Wild-type (left) and transgenic (right) littermates, with obvious fluid accumulation in the K_v4.2N mouse. B and C, Gross appearance of a wild-type (B) and transgenic (C) heart at 12 weeks of age. The cardiac contour is rounded and the left atrium is markedly enlarged in the transgenic mouse. D, A long-axis view of a transgenic heart demonstrates the chamber dilatation, including a markedly dilated left atrium that is filled with an organized thrombus. E and F, Short-axis view at the papillary muscle level of hematoxylin and eosin–stained frozen section of a wild-type (E) and a transgenic (F) heart, showing evidence of ventricular hypertrophy and chamber dilatation. G through I, Higher-power views of formalin-fixed and paraffin-embedded sections from matched control (G) and transgenic (H and I) ventricular myocardium, showing myocyte cell loss, cellular hypertrophy, vacuolization, interstitial fibrosis, and increased interstitial cellularity. J, Higher-power view of the left atrial thrombus seen in virtually all transgenic hearts.

TABLE 1. Morphological Properties of Mouse Hearts

	3 Weeks		12 to 14 Weeks	
	Control	Transgenic	Control	Transgenic
Body weight, g	10.4±0.4 (14)	10.5±0.6 (8)	21.6±0.8 (8)	21.2±1.3 (9)
Heart weight, mg	68±3 (14)	68±4 (8)	112±4 (8)	173±5* (9)
Heart/body, mg/g	6.6±0.2 (14)	6.5±0.2 (8)	5.2±0.1 (8)	8.4±0.8* (9)
Lung/body, mg/g	7.3±0.3 (5)	7.1±0.4 (4)	6.6±0.1 (4)	10.4±1.2* (4)
Liver/body, mg/g	46.1±0.4 (5)	43.6±0.5 (4)	42.0±0.4 (4)	48.5±5.3 (4)

Number of individual mice studied is shown in parentheses.
*$P<0.05$ between age-matched transgenic and nontransgenic mice.

tion was observed in essentially all transgenic mice at this stage (Figure 3J).

Phenotypic Characterization of 2- to 3.5-Week-Old Transgenic Mice

To begin to understand the time course of disease progression in the MHCα-K_v4.2N transgenic mice, we first characterized cardiac morphology, gene expression, ionic currents, and hemodynamic properties in neonatal preparations. At 2 to 3.5 weeks of age, despite robust transgene expression (Figure 2), there was no overt evidence of cardiac hypertrophy (see Table 1). Heart weights and heart weight/body weight ratios were indistinguishable from those of nontransgenic littermates. There was a modest increase in ventricular ANF expression but no significant alterations in other traditional markers of cardiac hypertrophy, including SERCA and PLB, as summarized in Table 2. In fact, average myocyte membrane capacitance (C_M) was significantly smaller ($P<0.04$) in transgenic myocytes (C_M=87.0±3.8 pF, n=33) compared with nontransgenic control cells (C_M=107.5±4.8 pF, n=27), consistent with the absence of gross cardiac enlargement.

We next examined the electrophysiological properties of myocytes from young transgenic mice and nontransgenic littermates. Figure 4A shows representative current density traces in right ventricular myocytes from 23-day-old control (left) and transgenic (center and right) littermates; Figure 4B shows the corresponding current-voltage relationships of the peak current (■) and the current remaining at the end of the 500-ms pulse (□) (ie, I_{500}) for the same cells. The difference between the peak outward current and the current remaining at the end of the 500-ms pulse was defined as the transient outward current (ie, I_{to}). The frequency histogram of I_{to} shown in Figure 4C demonstrates uniformly high current densities in control myocytes, with only 11.5% of these cells exhibiting I_{to} densities below 40 pA/pF. In contrast, transgenic myocytes displayed a much broader range of I_{to} densities, and almost half (45.4%) exhibited I_{to} densities below 40 pA/pF. Statistical comparisons based on χ^2 tests (see Materials and Methods) revealed that the 2 distributions were statistically distinct ($P<0.05$). Moreover, bivariate normal distribution functions gave statistically better fits ($P<0.05$, F statistics) to the current amplitude distribution data than a monovariate function in the transgenic mice but not in the control mice. These observations suggest that the current densities in transgenic mice comprise 2, or possibly more, populations of cells (see Discussion). Overall, at this age, I_{to} current densities were significantly ($P<0.05$) reduced in transgenic myocytes (48.7±5.4 pA/pF, n=33) compared with control myocytes (61.2±4.3 pA/pF, n=26), as assessed using the nonparametric Kolmogorov-Smirnov method. Identical observations were made from myocytes isolated from the left ventricle, where I_{to} measured at +60 mV was significantly ($P<0.02$) reduced from 57.4±4.3 pA/pF (n=22) in control hearts to 39.6±3.1 pA/pF (n=25) in transgenic hearts.

TABLE 2. Gene Expression

	3 Weeks		12 to 14 Weeks	
	Control	Transgenic	Control	Transgenic
ANF	1±0.23 (5)	2.74±0.48* (6)	1±5.47 (5)	42.7±4.99* (6)
SERCA	1±0.11 (5)	1.55±0.23 (6)	1±0.13 (5)	0.56±0.12* (6)
PLB	1±0.07 (5)	1.26±0.23 (6)	1±0.07 (5)	0.34±0.06* (6)
K_v4.2	1±0.14 (5)	1.30±0.11 (6)	1±0.83 (5)	3.77±0.74* (6)

Number of individual mice studied is shown in parentheses.
*$P<0.05$ between age-matched transgenic and control mice.

Next, we investigated whether changes in I_{to} were associated with alterations in action potential duration (APD). Control action potentials were typically short and showed no evidence of a plateau (Figure 4D, left). On average, control APD_{50} and APD_{90} values were 3.5±0.16 ms (n=12) and 20.6±2.1 ms (n=12). As with I_{to}, some myocytes from young transgenic mice had spiked action potentials that were indistinguishable from those of control cells (Figure 4D, center), whereas other myocytes had markedly prolonged action potentials that were never seen in controls (Figure 4D, right). Importantly, the myocytes with reduced I_{to} invariably had prolonged action potentials, consistent with I_{to} reduction being responsible for APD prolongation. Despite mixed populations of myocytes in transgenic mice, the average APD_{50} and APD_{90} values were significantly ($P<0.03$) prolonged in transgenic mice compared with control mice. Consistent with these observations, we found that monophasic action potentials recorded from the apical LV epicardium of transgenic hearts were significantly prolonged at both 50% and 90% of repolarization, as shown in Figure 5. APD_{50} was 12.3±1.0 ms in control versus 49.2±8.3 ms in transgenic hearts ($P<0.003$), and APD_{90} was 53.5±5.9 ms in control versus 108.7±11.0 ms in transgenic hearts ($P<0.005$), establishing that global changes in repolarization occur in young transgenic mice. Despite changes in APD, no differences in resting membrane potential between the groups (−83.8±0.9

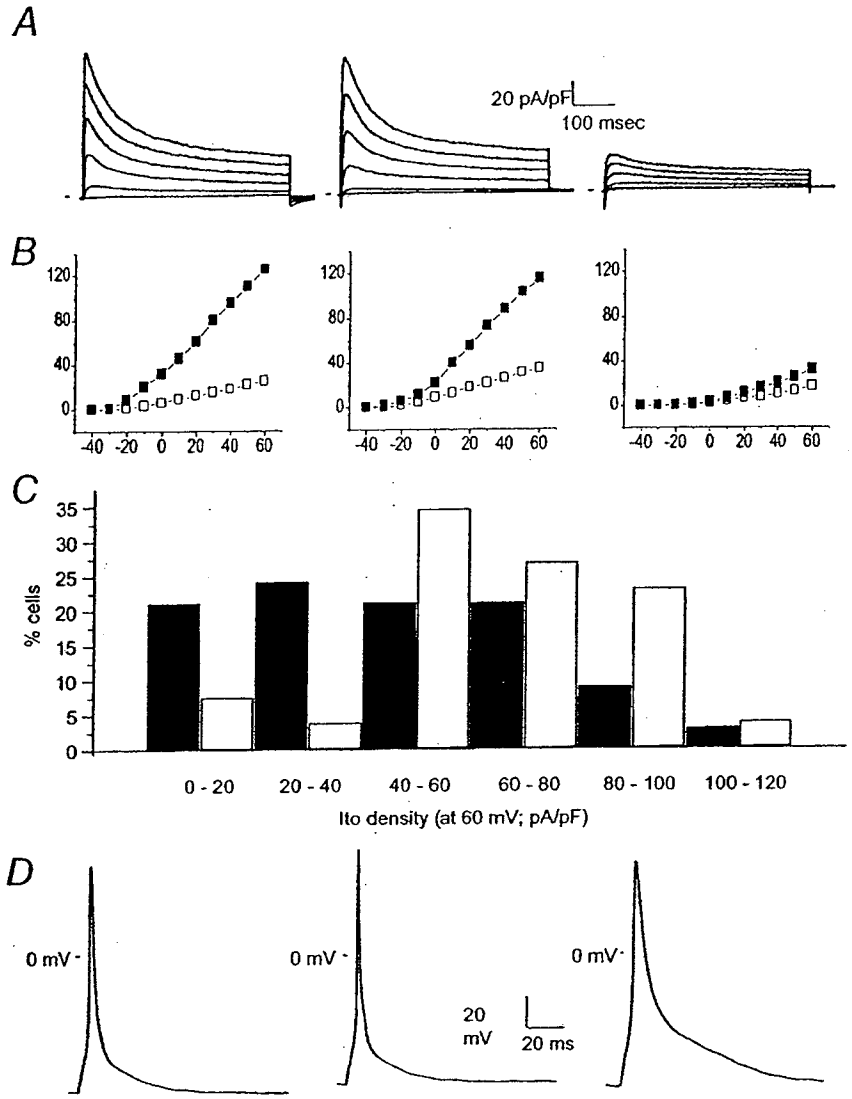


Figure 4. K^+ currents in 2- to 3.5-week-old mice. A, Representative outward current traces from a right ventricular myocyte from a 23-day-old control mouse (left) and from a transgenic littermate (center and right). Outward K^+ currents were induced by 500-ms depolarizations to a range of voltages (−40 to +60 mV in 10-mV steps) from a holding potential of −80 mV. Current traces have been normalized for cellular capacitance. Markers indicate 0 pA/pF. B, Current-voltage relationships, for the peak current (■) and the current remaining at the end of the 500-ms pulse (□), were constructed by plotting current density against step potential and are shown for control (left) and transgenic cells (center and right). Outward currents are reduced in some transgenic cells. C, Frequency distribution of I_{to} density in 26 control (open bars) and 33 transgenic (solid bars) cells. I_{to} density at this stage in control myocytes was uniformly high, whereas outward currents in a large subset of transgenic cells were clearly reduced. D, Typical representative action potentials from young control (left) and transgenic mice (center and right). Action potentials were typically spikelike in control mice, but APD was heterogeneous in the transgenic mice, with some action potentials similar to those of control mice (center) and others markedly prolonged (right). The action potentials shown in panel D were recorded from the same myocytes as those shown in panels A and B.

mV [n=12] and −83.7±0.7 mV [n=16], in control and transgenic cells, respectively) as expected from the I_{K1} results (see Figure 6).

To examine the specificity of the K_v4.2N-dependent reduction of I_{to}, several additional K^+ currents were examined. I_{500} (recorded at the end of a 500-ms depolarization step) and the distribution histograms were no different ($P>0.1$) in right ventricular myocytes from 2- to 3.5-week-old transgenic compared with control mice (I_{500}=24.4±1.3 pA/pF [n=26] control versus 21.5±1.2 pA/pF [n=31] transgenic). Similar results were observed in myocytes isolated from the left ventricle (not shown). Furthermore, I_{K1} densities were not significantly different between transgenic cells exhibiting I_{to} densities <40 pA/pF (I_{K1} density=−15.5±2.1 pA/pF, n=9) and control cells (−15.7±1.4, n=13) or transgenic cells exhibiting an I_{to} density above 40 pA/pF (−16.4±0.6 pA/pF, n=10), as illustrated in Figure 6. As expected, an ANCOVA on the distributions summarized in Figure 4C confirmed that I_{K1} densities did not correlate with I_{to} ($P>0.6$) (see online supplementary information for a tabulation of all electrophysiological parameters; http://www.circresaha.org).

We next examined the hemodynamic characteristics of these young mice. The mean aortic pressure and peak systolic ventricular pressures were significantly elevated in transgenic mice compared with age-matched control littermates. More notable were the significant increases in the magnitude of both +dP/dt and −dP/dt in the transgenic mice versus littermate controls. Neither heart rates nor end-diastolic pressures were significantly different between the groups, suggesting that the differences in dP/dt between the groups were not due to changes in preload. These differences in hemodynamic parameters are not the result of the limited frequency response of our pressure recording system, because signal filtering will tend to reduce (not enhance) the observed differences. The elevated contractility coincided with significant increases in both peak LV ejection velocities and fractional shortening, as measured by echocardiography using Doppler and M-mode recordings, respectively. These results, summarized in Table 3, establish unequivocally that contractility was elevated in the transgenic mice with reduced I_{to} and global action potential prolongation, before the development of overt hypertrophy.

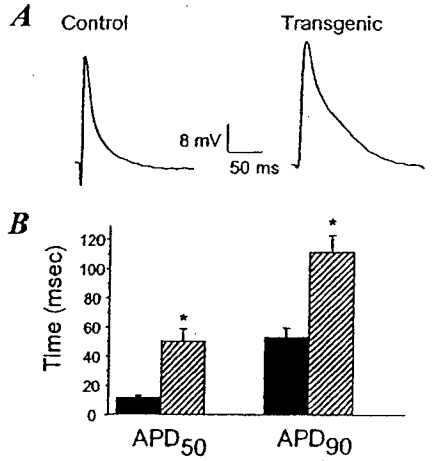


Figure 5. Monophasic action potential recordings. A, Typical monophasic action potential recordings from isolated hearts from control (left) and transgenic (right) mice. B, On average, APD_{50} and APD_{90} were significantly (*$P<0.01$) longer in the transgenic mouse hearts (hatched bars) vs control hearts (solid bars). Data are mean±SEM.

Phenotypic Characterization of Adult Mice

By 3 to 4 months of age, most transgenic mice developed clinical signs of congestive heart failure accompanied by gross and histological evidence of a cardiomyopathy (Figure 3). Hemodynamic studies confirmed this impression. Compared with littermate controls, transgenic mice had significantly ($P<0.05$) reduced peak aortic pressures, reduced peak systolic ventricular pressures, increased LV end-diastolic pressures, elevated heart rates, and severe depression of both +dP/dt and −dP/dt. Consistent with these hemodynamic changes, echocardiographic studies revealed that adult transgenic mice displayed significant reductions in LV fractional shortening and peak aortic injection velocities, as summarized in Table 3.

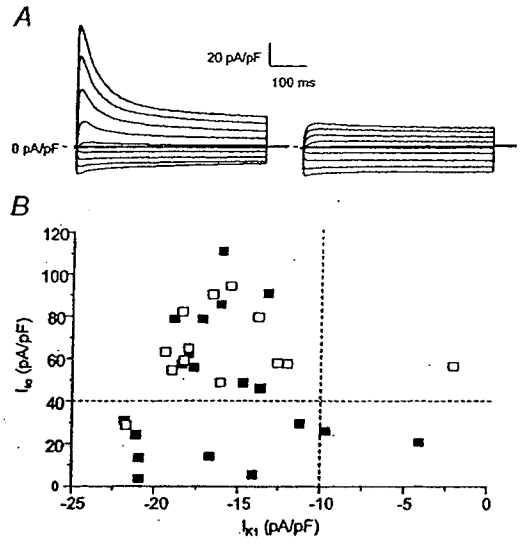


Figure 6. Inward rectifier currents in 2- to 3.5-week-old mice. A, Representative families of repolarizing K^+ currents recorded from myocytes isolated from 2- to 3.5-week-old control (left) and transgenic (right) mice. Currents were elicited by 500-ms steps to voltages in the range −130 to 60 mV (in 10-mV increments from −130 to −60 mV and then in 20-mV steps to 60 mV). I_{K1} densities were similar in the control and the transgenic cells, despite the fact that I_{to} was absent in the transgenic cells. B, I_{K1} density in control current (□) and transgenic (■) cells were determined at −130 mV and plotted against I_{to} density (at 60 mV) in the same cells. Cells below the dashed horizontal line and to the right of the dashed vertical line express low I_{to} density and low I_{K1} density, respectively (see text for explanation). ANCOVA of the distributions confirmed that low I_{K1} densities were not predictors of the density of I_{to}.

In contrast to the findings in young transgenic mice, the average myocyte membrane capacitance (C_M), estimated in patch-clamp experiments, was significantly ($P<0.05$) greater in transgenic cells (232±16 pF, n=16) compared with control cells (156±6 pF, n=23). Figure 7 shows representative current density traces (Figure 7A) and average current-voltage relationships (Figure 7B) recorded in control (left) and transgenic (right) right ventricular myocytes. Whereas I_{to} was activated over the same range of voltages between the 2 groups, the average current densities at +60 mV were reduced in transgenic (16.5±3.9 pA/pF, n=11) compared with control cells (48.4±3.4 pA/pF, n=21). As expected from the reductions in outward K^+ currents, action potentials were typically prolonged in myocytes isolated from the transgenic mice compared with age-matched control mice (Figure 7C). On average, APD_{50} and APD_{90} values were significantly ($P<0.05$) different between control (3.8±0.3 and 17.5±1.6 ms, n=8) and transgenic (7.7±0.7 and 87.9±19 ms, n=7) mice. Similar significant ($P<0.003$) reductions in I_{to} density and action potential prolongation were observed in myocytes isolated from the left ventricle of transgenic mice compared with nontransgenic controls (ie, I_{to} was 43.7±4.5 pA/pF [n=17] control versus 25.4±2.4 pA/pF [n=33] transgenic).

Finally, we examined whether the onset of cardiac hypertrophy and failure was accompanied by more global abnormalities in ionic currents or gene expression typical of diseased myocardium. Figure 8 shows representative inward rectifier (I_{K1}) current densities and current-voltage relationships in control and transgenic mice. The average Ba^{2+}-sensitive current-voltage relationships for control (■) and transgenic (●) mice, I_{K1}, was significantly ($P<0.005$) reduced. At −130 mV, I_{K1} was −14.8±1.0 pA/pF (n=12) and −6.5±0.7 pA/pF (n=8) in control and transgenic cells, respectively. In association with reductions in I_{K1}, transgenic myocytes had resting membrane potentials (−76.3±1.6 mV, n=7), which were significantly reduced ($P<0.05$) compared with control resting potentials (−82.3±1.8 mV, n=8).

Perturbations in cardiac gene expression were also evident at this stage. As illustrated in Figure 9 and summarized in Table 2, SERCA and PLB transcript abundance were both significantly reduced by 2- to 3-fold ($P<0.04$); βMHC gene expression was reinduced; and ANF levels were markedly increased, almost 50-fold ($P<0.01$), in transgenic mice compared with littermate controls. Despite the obvious reductions in I_{to} current shown in Figure 6, endogenous $K_v4.2$ message and protein were modestly increased in transgenic mice compared with controls.

Discussion

Previous studies in animal models and human patients have established that reductions in the Ca^{2+}-independent transient

TABLE 3. Hemodynamic and Echocardiographic Analysis

	2 to 3 Weeks		12 to 14 Weeks	
	Control	Transgenic	Control	Transgenic
Peak aortic pressure, mm Hg	72±2 (20)	85±5 (17)*	91±3 (9)	63±5 (7)*
Peak LV systolic pressure, mm Hg	73±3 (13)	89±4 (9)*	95±6 (5)	60±5 (6)*
LV end-diastolic pressure, mm Hg	3.6±0.7 (9)	3.3±0.7 (13)	3.0±0.8 (5)	20.2±2.1 (6)*
Peak +dP/dt, mm Hg/s	1956±83 (9)	2470±146 (13)*	3458±148 (4)	927±269 (6)*
Peak −dP/dt, mm Hg/s	−1777±98 (9)	−2284±135 (13)*	−3398±133 (4)	−709±142 (6)*
Heart rate, beats/min	287±11 (17)	267±12 (20)	234±14 (9)	350±19 (7)*
Fractional shortening	0.45±0.01 (7)	0.50±0.01 (7)*	0.45±0.05 (2)	0.24±0.02 (2)*
Peak aortic ejection velocity, m/s	0.80±0.022 (6)	0.90±0.04* (6)	0.92±0.03	0.69±0.03*

Number of individual mice studied is shown in parentheses.
*$P<0.05$ between age-matched transgenic and control mice.

outward current (I_{to}) are among the most common alterations in K^+ currents observed in heart disease.[11,13,38–40] In this study, we sought to understand whether alterations of ionic currents such as I_{to} in heart disease simply reflect global alterations in cardiac gene expression or whether these electrophysiological changes might contribute to the disease process. Members of the $K_v4.x$ family have previously been demonstrated to contribute to cardiac I_{to} in a number of species and to be downregulated in disease.[26,29,41–44] Accordingly, we targeted expression of a dominant-negative $K_v4.2N$ fragment to the heart using the αMHC promoter, with the expectation that such a strategy would specifically repress cardiac $K_v4.x$-dependent currents.[30] Generation of stable transgenic lineages harboring the $K_v4.2N$ transgene was difficult, immediately suggesting that expression of the truncated protein in the heart was poorly tolerated. Although our studies were therefore limited to observations in a single transgenic line, other investigators have reported a similar deleterious phenotype with $K_v4.2$ truncation mutants (G. Tomaselli, personal communication, November 1998), suggesting that the observed effect is specific to expression of the mutant protein and not a result of insertional mutagenesis.

Phenotypic evaluation of the MHCα-$K_v4.2N$ transgenic mice demonstrated progression from a hypercontractile state with normal cardiac morphology to one of profound myocardial hypertrophy, dysfunction, and failure. The enhanced contractility in young transgenic mice is likely a result of prolongation of the APD and the resulting effects on $[Ca^{2+}]_i$ transient amplitude. Indeed, $[Ca^{2+}]_i$ transients are elevated in transgenic myocytes from 2- to 3.5-week-old mice with prolonged action potentials compared with myocytes with normal action potential profiles (data not shown). Such a relationship between APD prolongation and $[Ca^{2+}]_i$ transients has previously been described in dissociated myocytes in several settings, including normal cells subjected to action potential clamp, myocytes from rats with experimental myocardial infarction or with spontaneous hypertension, as well as those transduced with adenoviral vectors expressing K^+ channel subunits.[21,35,45,46] However, this is the first report, to our knowledge, demonstrating that primary genetic manipulation of the action potential can influence cardiac contractility in vivo.

The mechanisms accounting for the progression from the hypercontractile phenotype in young mice to congestive heart failure in the older animals is unclear, although a number of possible explanations exist. A variety of kinases, phosphatases, and receptor signaling pathways contributing to myocyte growth and possibly apoptosis are directly activated by Ca^{2+} or use Ca^{2+} as an essential cofactor.[24,47,48] Thus, the development of heart disease and hypertrophy in these mice might conceivably be linked to sustained elevations in $[Ca^{2+}]_i$ secondary to APD prolongation.

Alternatively, the heart failure phenotype in older mice may not be directly linked to APD prolongation and elevated $[Ca^{2+}]_i$ but rather to unanticipated and indirect effects of the ectopically expressed mutant $K_v4.2N$ polypeptide. Recent studies have demonstrated that overexpression of foreign proteins can induce an endoplasmic reticular (ER) stress response, resulting in activation of a number of cell signaling and kinase pathways such as C/EBP homologous protein (CHOP) and the induction of apoptosis.[49,50] Consistent with this mechanism, previous studies in cultured myocytes expressing a truncated $K_v4.2$-GFP fusion construct[51] as well as GH3 pituitary cells expressing a truncated $K_v1.1$ polypeptide[52] have both demonstrated abnormal trapping of the mutant protein in the ER. Indeed, Huang and Izumo[53] have recently described a cardiomyopathy in transgenic mice expressing high levels of a "biologically inert" protein. Thus, it is conceivable that the truncated Kv4.2N polypeptide, which is expressed at levels ≈10-fold greater than the endogenous full-length protein, may induce a cardiomyopathy by interference with normal cell trafficking and the induction of an ER stress response. Consistent with this alternative mechanism, Barry et al[29] have reported that reducing I_{to} by overexpressing an alternative dominant-negative $K_v4.2$ polypeptide, which differs from the wild-type protein by only a single point mutation in the pore region (W362F), is not accompanied by obvious hypertrophy or overt heart disease. The discordant phenotype between these 2 models for I_{to} reduction might originate from differences in the timing and/or level of transgene expression or differences in genetic backgrounds. Unfortunately, these other investigators did not assess contractile function or $[Ca^{2+}]_i$ transients in their transgenic mice to determine whether they also show an

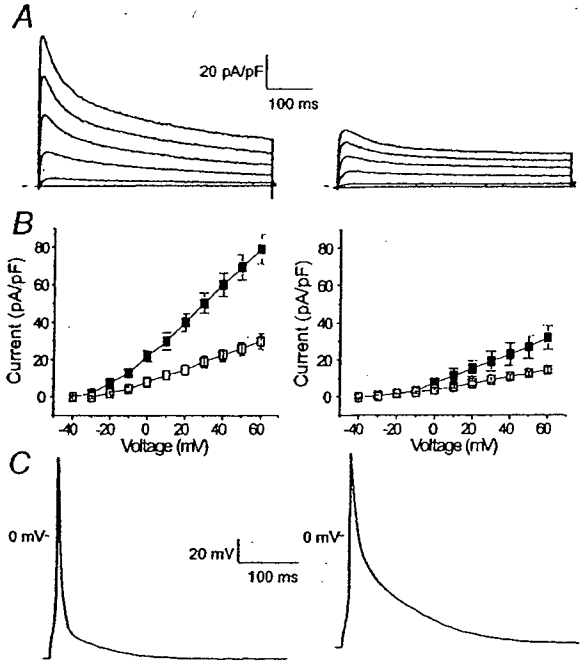


Figure 7. K^+ currents and action potentials in 12- to 13-week-old mice. A, Representative current traces recorded from a right ventricular myocyte isolated from a 12-week-old control mouse (left) and transgenic littermate (right). Outward K^+ currents were induced by 500-ms depolarizations to a range of voltages (−40 to +60 mV in 10-mV steps) from a holding potential of −80 mV. Prepulses to −40 mV for 30 ms were used to eliminate Na^+ currents, whereas I_{Ca} was blocked by adding 0.3 mmol/L extracellular $CdCl_2$. Note that current traces have been normalized for cellular capacitance to control for differences in cell size. Markers indicate 0 pA/pF. B, Average current-voltage relationships showing the current density at the peak (■) and at the end of the 500-ms depolarizing pulse (□), plotted as a function of the membrane potential for control (left) and transgenic (right) myocytes. Symbols represent mean values derived from 11 to 21 cells. Data are mean±SEM. Note that both the difference between the peak outward current and the current remaining at the end of the 500-ms pulse (ie, I_{to}) and current remaining at the end of the 500-ms pulse were reduced in 12- to 13-week-old transgenic mice. C, Representative action potentials from 12- to 13-week-old control (left) and transgenic (right) mice. Action potentials were greatly prolonged in transgenic myocytes.

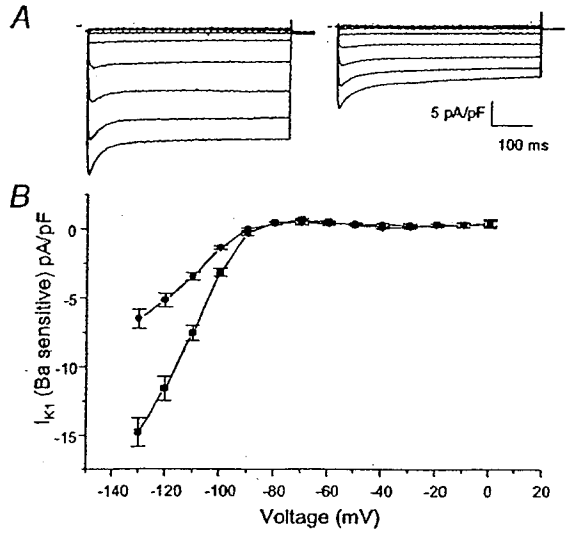


Figure 8. Inward rectifier currents in 12- to 13-week-old mice. A, Representative inward rectifier (I_{K1}) currents recorded from myocytes isolated from 12- to 13-week-old control (left) and transgenic (right) mice. All currents were normalized to cell capacitance (pA/pF) to correct for differences in cell size. B, Average I_{K1} current-voltage relationships in control (■) and transgenic (●) mice. I_{K1} current density was significantly reduced in transgenic compared with control myocytes. Data are mean±SEM derived from 8 to 12 cells.

enhanced contractile state. Clearly, additional studies will be necessary to reconcile these varying phenotypes.

Surface ECGs of anesthetized mice did not disclose convincing evidence of QT prolongation in the $K_v4.2N$ transgenic mice at any stage, despite prolongation of monophasic action potentials in situ and APD prolongation in a significant proportion of isolated myocytes. However, several genetic models in which cardiac repolarizing currents are reduced, including loss of function of minK[54,55] and dominant-negative inhibition of ERG,[56] result in no gross perturbations of the surface ECG in anesthetized mice. Given the difficulty of precisely identifying intervals on the mouse surface ECG,[57] the monophasic action potentials may more accurately reflect the time course of repolarization and are certainly consistent with the significant depression of I_{to}.

Clinically, prolongation of the cardiac action potential has been associated with an increased propensity for cardiac arrhythmias, particularly when heterogeneous in nature. Although death was typically sudden in the $K_v4.2N$ transgenic mice, even in those without obvious signs of congestion, short-term electrocardiographic recordings in anesthetized mice did not reveal the presence of ventricular arrhythmias. We and others have recently used telemetric methodology for long-term electrocardiographic recordings of fully conscious genetically modified mice.[58] Clearly, further studies to systematically determine the propensity for spontaneous or inducible arrhythmogenesis are warranted.

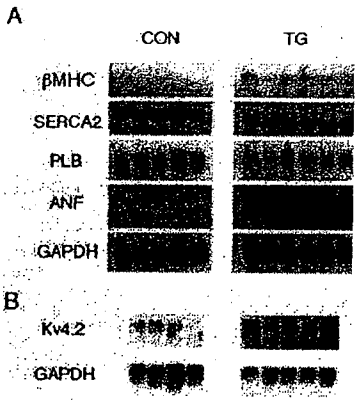


Figure 9. Molecular markers of hypertrophy in $K_v4.2N$ transgenic mice. Northern blot analysis of total RNA (A) or mRNA (B) from the ventricles of age-matched control (CON) and transgenic (TG) mice. Membranes were hybridized with probes for βMHC, SERCA, PLB, ANF, endogenous $K_v4.2$, and the transgene (MHC-$K_v4.2N$) and loading assessed with a probe for GAPDH. Autoradiographic signals were quantified using NIH Image (version 1.59) and normalized to the GAPDH signal. Transgenic hearts showed significant reductions in SERCA and PLB expression, modest induction of βMHC, and marked induction of ANF (see Table 2). Endogenous $K_v4.2N$ expression was also modestly increased.

The extent to which our results are applicable to other species is uncertain. Patients with congenital long-QT syndrome rarely show evidence of cardiac hypertrophy,[19] suggesting that the effects of I_{to} reduction in our mice might not be applicable to humans. However, all previously identified channel mutations associated with long-QT syndrome affect action potential profiles in a manner that is distinct from changes produced by I_{to} reductions and therefore might not produce equivalent effects. Indeed, we have found that prolongation in the early repolarization period has a much greater effect on $[Ca^{2+}]_i$ than late in repolarization (data not shown). Regardless, to date, no forms of congenital long-QT syndrome have been linked to K^+ channels producing transient outward currents.[19,20]

In summary, we have generated transgenic mice expressing a dominant-negative N-terminal fragment of the $K_v4.2$ potassium channel subunit in the heart. Young transgenic mice show heterogeneous reductions in I_{to} and APD prolongation, in association with a hypercontractile state. Between 8 and 16 weeks of age, these mice develop a dilated cardiomyopathy with profound cardiac dysfunction, culminating in congestive heart failure and death. Although the mechanism(s) by which ectopic expression of the $K_v4.2N$ protein in the heart leads to cardiac hypertrophy and failure remains to be clarified, our working hypothesis is that reductions in I_{to} and consequent APD prolongation, at least in the rodent, lead to the development of cardiac hypertrophy and heart failure through alterations in calcium delivery and activation of downstream signaling cascades.

Acknowledgments

This work was supported by the Heart & Stroke Foundation of Canada (P.H.B.) and the American Heart Association, National Center and Heritage Affiliate (G.I.F.). P.H.B. holds a Medical Research Council of Canada scholarship award. G.I.F. is an Established Investigator of the American Heart Association. Equipment support from the Alan Tiffin Trust Fund and The Center for Cardiovascular Research is gratefully acknowledged. We thank M. Tamkun for providing the $K_v1.4$ clone, R. Joho for providing the $K_v2.1$ clone, J. Nerbonne and L. Jan for providing $K_v4.2$ clones, and G. Tomaselli for sharing unpublished data. We also thank Zamaneh Kassiri, Roger Kaprielian, and especially Tin Nguyen for assistance in these studies.

References

1. Watkins H, Seidman JG, Seidman CE. Familial hypertrophic cardiomyopathy: a genetic model of cardiac hypertrophy. *Hum Mol Genet.* 1995; 4:1721–1727.
2. Seidman CE, Seidman JG. Mutations in cardiac myosin heavy chain genes cause familial hypertrophic cardiomyopathy. *Mol Biol Med.* 1991; 8:159–166.
3. Ledford DK. Immunologic aspects of vasculitis and cardiovascular disease. *JAMA.* 1997;278:1962–1971.
4. Deedwania PC. The progression from hypertension to heart failure. *Am J Hypertens.* 1997;10:280S–288S.
5. Pfeffer MA, Pfeffer JM, Lamas GA. Development and prevention of congestive heart failure following myocardial infarction. *Circulation.* 1993;87(suppl IV):IV-120–IV-125.
6. Bassand JP. Left ventricular remodelling after acute myocardial infarction–solved and unsolved issues. *Eur Heart J.* 1995;16(suppl I):58–63.
7. Anversa P, Olivetti G, Capasso JM. Cellular basis of ventricular remodeling after myocardial infarction. *Am J Cardiol.* 1991;68:7D–16D.
8. Colucci WS. Molecular and cellular mechanisms of myocardial failure. *Am J Cardiol.* 1997;80:15L–25L.
9. Sadoshima J, Izumo S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol.* 1997;59:551–571.
10. Bohm M, Flesch M, Schnabel P. Beta-adrenergic signal transduction in the failing and hypertrophied myocardium. *J Mol Med.* 1997;75: 842–848.
11. Beuckelmann DJ, Nabauer M, Erdmann E. Alterations of K^+ currents in isolated human ventricular myocytes from patients with terminal heart failure. *Circ Res.* 1993;73:379–385.
12. Qin D, Zhang ZH, Caref EB, Boutjdir M, Jain P, el-Sherif N. Cellular and ionic basis of arrhythmias in postinfarction remodeled ventricular myocardium. *Circ Res.* 1996;79:461–473.
13. Kaab S, Nuss HB, Chiamvimonvat N, O'Rourke B, Pak PH, Kass DA, Marbán E, Tomaselli GF. Ionic mechanism of action potential prolongation in ventricular myocytes from dogs with pacing-induced heart failure. *Circ Res.* 1996;78:262–273.
14. Koumi S, Backer CL, Arentzen CE. Characterization of inwardly rectifying K^+ channel in human cardiac myocytes: alterations in channel behavior in myocytes isolated from patients with idiopathic dilated cardiomyopathy. *Circulation.* 1995;92:164–174.
15. Gidh-Jain M, Huang B, Jain P, el-Sherif N. Differential expression of voltage-gated K^+ channel genes in left ventricular remodeled myocardium after experimental myocardial infarction. *Circ Res.* 1996;79: 669–675.
16. Roden DM. Ionic mechanisms for prolongation of refractoriness and their proarrhythmic and antiarrhythmic correlates. *Am J Cardiol.* 1996;78: 12–16.
17. Swynghedauw B, Chevalier B, Charlemagne D, Mansier P, Carre F. Cardiac hypertrophy, arrhythmogenicity and the new myocardial phenotype, II: the cellular adaptational process. *Cardiovasc Res.* 1997;35:6–12.
18. Antzelevitch C, Nesterenko VV, Yan GX. Role of M cells in acquired long QT syndrome, U waves, and torsade de pointes. *J Electrocardiol.* 1995;28:131–138.
19. Wilde AA, Veldkamp MW. Ion channels, the QT interval, and arrhythmias. *Pacing Clin Electrophysiol.* 1997;20:2048–2051.
20. Kass RS, Davies MP. The roles of ion channels in an inherited heart disease: molecular genetics of the long QT syndrome. *Cardiovasc Res.* 1996;32:443–454.
21. Bouchard RA, Clark RB, Giles WR. Effects of action potential duration on excitation-contraction coupling in rat ventricular myocytes: action potential voltage-clamp measurements. *Circ Res.* 1995;76:790–801.
22. Fedida D, Bouchard RA. Mechanisms for the positive inotropic effect of alpha 1-adrenoceptor stimulation in rat cardiac myocytes [published correction appears in *Circ Res.* 1993;72:489]. *Circ Res.* 1992;71:673–688.
23. Nagashima M, Hattori Y, Akaishi Y, Tohse N, Sakuma I, Kitabatake A, Kanno M. Alpha 1-adrenoceptor subtypes mediating inotropic and electrophysiological effects in mammalian myocardium. *Am J Physiol.* 1996; 271:H1423–H1432.
24. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell.* 1998;93:215–228.
25. Barry DM, Trimmer JS, Merlie JP, Nerbonne JM. Differential expression of voltage-gated K^+ channel subunits in adult rat heart: relation to functional K^+ channels? *Circ Res.* 1995;77:361–369.
26. Dixon JE, McKinnon D. Quantitative analysis of potassium channel mRNA expression in atrial and ventricular muscle of rats. *Circ Res.* 1994;75:252–260.
27. Fiset C, Clark RB, Shimoni Y, Giles WR. Shal-type channels contribute to the Ca2+-independent transient outward K^+ current in rat ventricle. *J Physiol (Lond).* 1997;500:51–64.
28. Wickenden AD, Kaprielian R, Parker TG, Jones OT, Backx PH. Effects of development and thyroid hormone on K^+ currents and K^+ channel gene expression in rat ventricle. *J Physiol (Lond).* 1997;504:271–286.
29. Barry DM, Xu H, Schuessler RB, Nerbonne JM. Functional knockout of the transient outward current, long-QT syndrome, and cardiac remodeling in mice expressing a dominant-negative Kv4 α subunit. *Circ Res.* 1998; 83:560–567.
30. Li M, Jan YN, Jan LY. Specification of subunit assembly by the hydrophilic amino-terminal domain of the Shaker potassium channel. *Science.* 1992;257:1225–1230.
31. De Leon JR, Federoff HJ, Dickson DW, Vikstrom KL, Fishman GI. Cardiac and skeletal myopathy in beta myosin heavy-chain simian virus 40 tsA58 transgenic mice. *Proc Natl Acad Sci U S A.* 1994;91:519–523.
32. Passman RS, Fishman GI. Regulated expression of foreign genes in vivo after germline transfer. *J Clin Invest.* 1994;94:2421–2425.

33. Tsushima RG, Li RA, Backx PH. P-loop flexibility in Na+ channel pores revealed by single- and double-cysteine replacements. *J Gen Physiol.* 1997;110:59–72.
34. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 1981;391:85–100.
35. Kaprielian RA, Wickenden AD, Kassiri Z, Parker TG, Liu PP, Backx PH. Relationship between K^+ channel down-regulation and $[Ca^{2+}]_i$ in rat ventricular myocytes following myocardial infarction. *J Physiol (Lond).* 1999;517:229–245.
36. Franz MR. Current status of monophasic action potential recording: theories, measurements and interpretations. *Cardiovasc Res.* 1999;41: 25–40.
37. Xu J, Yu W, Jan YN, Jan LY, Li M. Assembly of voltage-gated potassium channels: conserved hydrophilic motifs determine subfamily-specific interactions between the α-subunits. *J Biol Chem.* 1995;270:24761–24768.
38. Ryder KO, Bryant SM, Hart G. Membrane current changes in left ventricular myocytes isolated from guinea pigs after abdominal aortic coarctation. *Cardiovasc Res.* 1993;27:1278–1287.
39. Brooksby P, Levi AJ, Jones JV. The electrophysiological characteristics of hypertrophied ventricular myocytes from the spontaneously hypertensive rat. *J Hypertens.* 1993;11:611–622.
40. Takimoto K, Li D, Hershman KM, Li P, Jackson EK, Levitan ES. Decreased expression of Kv4.2 and novel Kv4.3 K^+ channel subunit mRNAs in ventricles of renovascular hypertensive rats. *Circ Res.* 1997; 81:533–539.
41. Dixon JE, Shi W, Wang HS, McDonald C, Yu H, Wymore RS, Cohen IS, McKinnon D. Role of the Kv4.3 K^+ channel in ventricular muscle: a molecular correlate for the transient outward current [published correction appears in *Circ Res.* 1997;80:147]. *Circ Res.* 1996;79:659–668.
42. Matsubara H, Suzuki J, Inada M. Shaker-related potassium channel, Kv1.4, mRNA regulation in cultured rat heart myocytes and differential expression of Kv1.4 and Kv1.5 genes in myocardial development and hypertrophy. *J Clin Invest.* 1993;92:1659–1666.
43. Brahmajothi MV, Morales MJ, Rasmusson RL, Campbell DL, Strauss HC. Heterogeneity in K^+ channel transcript expression detected in isolated ferret cardiac myocytes. *Pacing Clin Electrophysiol.* 1997;20: 388–396.
44. Brahmajothi MV, Morales MJ, Liu S, Rasmusson RL, Campbell DL, Strauss HC. In situ hybridization reveals extensive diversity of K^+ channel mRNA in isolated ferret cardiac myocytes. *Circ Res.* 1996;78: 1083–1089.
45. Brooksby P, Levi AJ, Jones JV. Investigation of the mechanisms underlying the increased contraction of hypertrophied ventricular myocytes isolated from the spontaneously hypertensive rat. *Cardiovasc Res.* 1993;27:1268–1277.
46. Nuss HB, Johns DC, Kaab S, Tomaselli GF, Kass D, Lawrence JH, Marbán E. Reversal of potassium channel deficiency in cells from failing hearts by adenoviral gene transfer: a prototype for gene therapy for disorders of cardiac excitability and contractility. *Gene Ther.* 1996;3: 900–912.
47. Sadoshima J, Izumo S. Signal transduction pathways of angiotensin II-induced c-fos gene expression in cardiac myocytes in vitro: roles of phospholipid-derived second messengers. *Circ Res.* 1993;73:424–438.
48. Finkbeiner S, Greenberg ME. Ca(2+)-dependent routes to Ras: mechanisms for neuronal survival, differentiation, and plasticity? *Neuron.* 1996;16:233–236.
49. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* 1998;12:982–995.
50. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev.* 1999;13:1211–1233.
51. Johns DC, Nuss HB, Marbán E. Suppression of neuronal and cardiac transient outward currents by viral gene transfer of dominant-negative Kv4.2 constructs. *J Biol Chem.* 1997;272:31598–31603.
52. Folco E, Mathur R, Mori Y, Buckett P, Koren G. A cellular model for long QT syndrome: trapping of heteromultimeric complexes consisting of truncated Kv1.1 potassium channel polypeptides and native Kv1.4 and Kv1.5 channels in the endoplasmic reticulum. *J Biol Chem.* 1997;272: 26505–26510.
53. Huang W-Y, Izumo S. Transgenic overexpression of a "biologically inert" molecule can caused dilated cardiomyopathy: a caveat in cardiac transgenic mice. American Heart Association's Scientific Conference on Molecular, Cellular, and Integrated Physiological Approaches to the Failing Heart, Snowbird, Utah, August 1999.
54. Drici MD, Arrighi I, Chouabe C, Mann JR, Lazdunski M, Romey G, Barhanin J. Involvement of IsK-associated K^+ channel in heart rate control of repolarization in a murine engineered model of Jervell and Lange-Nielsen syndrome. *Circ Res.* 1998;83:95–102.
55. Kupershmidt S, Yang T, Anderson ME, Wessels A, Niswender KD, Magnuson MA, Roden DM. Replacement by homologous recombination of the minK gene with lacZ reveals restriction of minK expression to the mouse cardiac conduction system. *Circ Res.* 1999;84:146–152.
56. Babij P, Askew GR, Nieuwenhuijsen B, Su CM, Bridal TR, Jow B, Argentieri TM, Kulik J, DeGennaro LJ, Spinelli W, Colatsky TJ. Inhibition of cardiac delayed rectifier K^+ current by overexpression of the long-QT syndrome HERG G628S mutation in transgenic mice. *Circ Res.* 1998;83:668–678.
57. Mitchell GF, Jeron A, Koren G. Measurement of heart rate and Q-T interval in the conscious mouse. *Am J Physiol.* 1998;274:H747–H751.
58. Lee P, Morley G, Huang Q, Fischer A, Seiler S, Horner JW, Factor S, Vaidya D, Jalife J, Fishman GI. Conditional lineage ablation to model human diseases. *Proc Natl Acad Sci USA.* 1998;95:11371–11376.

EXHIBIT K

Gene Therapy (1999) 6, 683–688

http://www.stockton-press.co.uk/gt

BRIEF COMMUNICATION

Gene delivery to the myocardium by intrapericardial injection

Y Fromes[1], A Salmon[1], X Wang[1], H Collin[1], A Rouche[1], A Hagège[2], K Schwartz[1] and MY Fiszman[1]
[1]*INSERM U153 – Institut de Myologie, Paris; and* [2]*Laboratoire de recherche en imagerie, Faculté de Médecine Necker-Enfants Malades, Université Paris V, Paris, France*

Several studies have demonstrated the feasibility of gene transfer into the heart muscle. However, all the available data also indicate that the extent of transfection remains limited. As an alternative method to intravascular administration, we have developed a novel strategy which uses the pericardial sac. When a replication-deficient adenovirus containing the cDNA encoding a bacterial β-galactosidase is injected into the pericardial sac of adult Wistar rats the staining is exclusively restricted to the pericardial cell layers. However, injecting a mixture of collagenase and hyaluronidase together with the virus, leads to a large diffusion of the transgene activity, reaching up to 40% of the myocardium. Transgene expression is predominant in the left ventricle and the interventricular septum but limited in the right ventricle. In vivo echocardiographic measurements of the left ventricular diameters at end diastolic and end systolic times show no difference between virus- and sham-injected animals, thus indicating a good clinical tolerance to this strategy of virus delivery. The same protocol has been used with the same efficiency in mice, which leads us to propose injection into the pericardial sac as an effective and harmless method for gene transfer into the heart muscle.

Keywords: *gene transfer; gene therapy; pericardium; adenovirus; myocardium*

Gene therapy is a potential new strategy for cardiovascular diseases which concerns single gene disease as well as more complex multigene pathologies affecting either the heart muscle or its vasculature. One of the major issues raised by this strategy is the development of efficient and safe gene transfer techniques.

So far, a number of publications have demonstrated the potential of direct injection of genes into the myocardium.[1–7] This may seem an attractive approach as it avoids complicated and expensive *ex vivo* manipulations followed by *in vivo* cell or organ grafting. However, direct injection into the myocardium results in gene expression over a limited region due to the lack of diffusion of the vector and, furthermore, it also results in local tissue damage which makes it a rather inappropriate therapeutic approach.[4,5,8] Intravascular gene delivery has raised higher hopes since the myocardium has a high density of capillaries.[10,11] Indeed, as opposed to single shot injection, it has been proved that coronary infusion allows some diffusion of reporter genes to the myocardium. However, at the present state of our knowledge, the efficiency of this strategy appears to be still limited by various factors such as a low permeability of the capillaries to large molecules or particles due to their continuous non fenestrated endothelium, or to the need for high perfusion pressure or because of a limited time contact of the vector with the target cell.[12] Moreover, Wright *et al*[13] pointed out that intracoronary infusion of cationic liposomes can result in multiple microinfarction.

In view of these difficulties, we have developed an alternative approach for myocardial gene transfer which is based on local delivery of the therapeutic gene into the pericardium.

The pericardial envelope of the heart may be used to overcome some of the restrictions inherent in other transfer methods. First, access to the pericardium can be obtained through rather simple procedures. Second, since the pericardium has a closed cavity, this may be used to maintain a prolonged contact time with the genetic vectors. The drawback of this approach is that the pericardial envelope appears to be rather tight, however, we will show that diffusion across this membrane can be increased by various pharmacological agents. Using this simple procedure, we show that significant territories of the myocardium can be transduced by adenoviral vectors.

Adult male Wistar rats were injected with a ΔE1-recombinant adenoviral vector containing a CMV-driven bacterial β-galactosidase gene without a nuclear localization signal.[14] We used adenovirus since it has proven to be a rather reliable vector for gene transfer into the heart muscle due to its ability to infect nondividing cells, an absolute requirement for terminally differentiated cardiac myocytes.[9] Intra-pericardial injections were performed through a small incision of the abdominal wall below the xyphoid appendix, with a trans-diaphragmatic approach. Delivery of adenoviral vectors into the pericardium can be performed safely under visual control. Seven days

Correspondence: Y Fromes, INSERM U153 – Institut de Myologie, Bâtiment Babinski – Hôpital Pitie-Salpetrière, 47 boulevard de l'Hôpital, 75651 Paris Cedex 13, France
Received 12 August 1998; accepted 4 November 1998

after the injection, the animals were killed and their hearts as well as other organs (lungs, diaphragm, liver, kidney, spleen) were harvested. Serial frozen sections were performed and assayed for β-galactosidase activity. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining was observed almost exclusively in the pericardial cell layers (Figure 1). Only rarely were myocardial cells found to be transduced by the adenovirus (Figure 1b). No significant staining was found either in the lungs or the diaphragm (data not shown). The same observation was also true for organs at the abdominal level, such as the liver, spleen or kidneys. Hence, the pericardium appears to be transduced by adenoviral vectors, but it also proves to be a tight envelope around the heart which allows no significant transduction of the myocardium. A similar observation was made by another group.[15] Such data are not so surprising since pericardial cells form a layer of tightly joined cells limiting diffusion across this anatomical barrier. Moreover, the connective tissue layers found in the cardiac muscle most probably diminish the spread and overall infectivity of vectors.[16–18] Various strategies have been described to circumvent this problem. Lamping *et al*[15] showed that pre-treatment with tetracycline did increase ventricular myocyte transfection, possibly through the induction of an inflammatory process which could have increased a vector uptake at the level of the epicardium. Interestingly, Aoki *et al*[19] have reported myocardial transfection after injection within the pericardium of a hemagglutinating virus of Japan (HVJ)–liposome complex. However, their data only showed transfection in a limited area of the myocardium which extended through few surface layers. Transfected cells included cardiac myocytes and fibroblasts beneath the pericardium and some myocytes in the middle of the myocar-

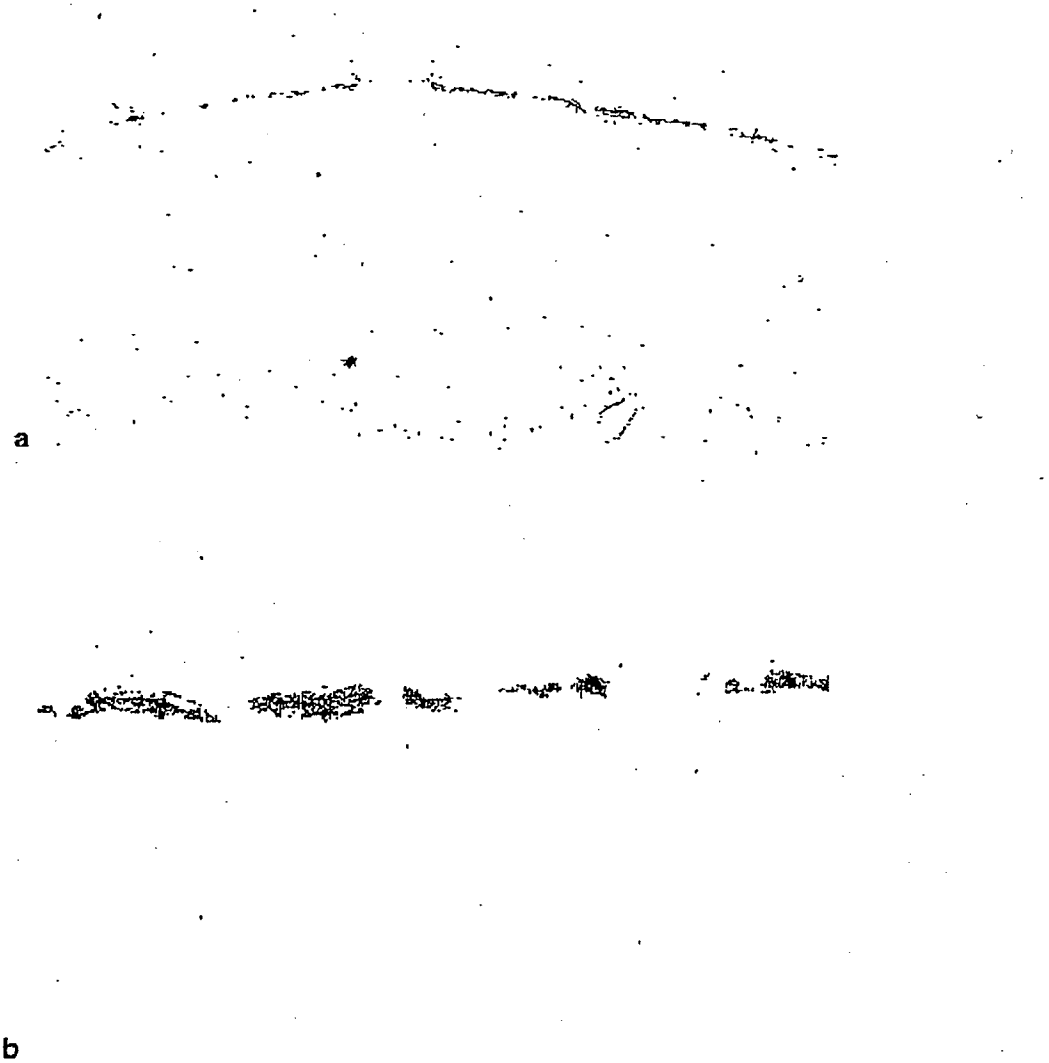


Figure 1 Intrapericardial administration of E1-deficient adenovirus containing a CMV-driven β-galactosidase gene. Wistar rats were injected locally with 4 × 10⁸ p.f.u. of recombinant adenovirus. The enzyme activity was revealed by incubation of cryosections in X-gal. (a) Low magnification (×40) shows transfection of the visceral pericardial cells. (b) Higher magnification (×100) reveals that the adenovirus did not transduce cardiac myocytes.

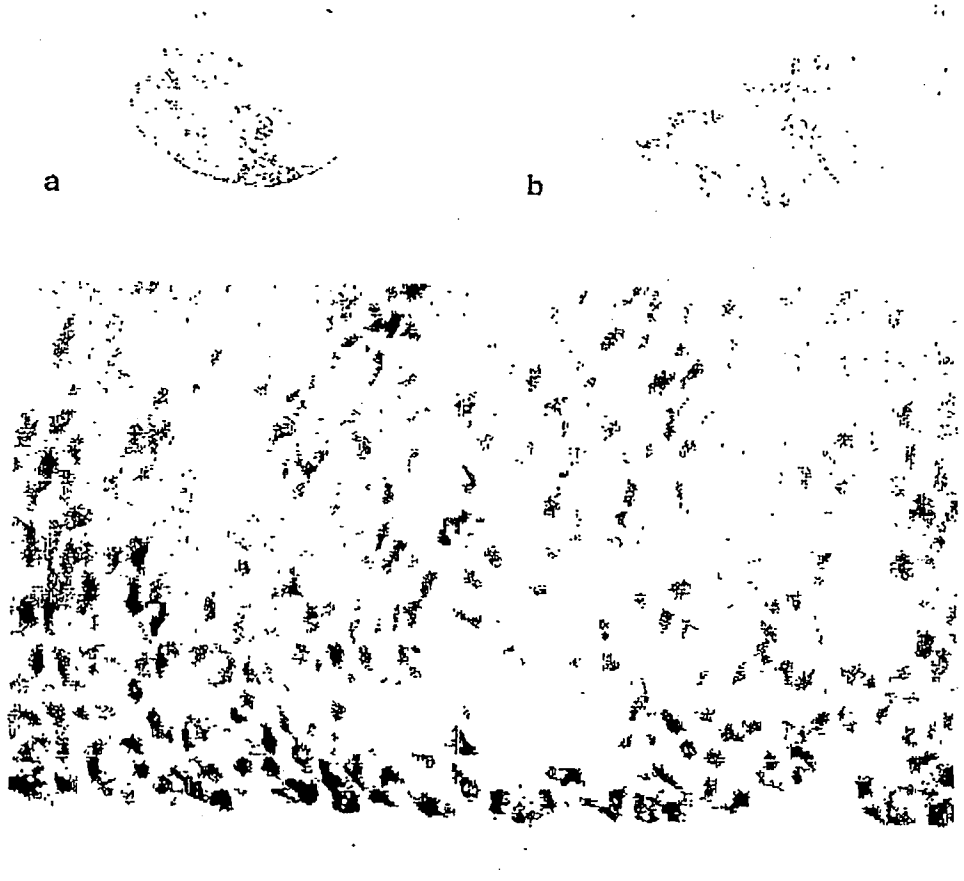


Figure 2 Intrapericardial vector delivery in the presence of proteolytic enzymes improves gene transfer. Serial cryosections demonstrate the extent of X-gal staining of the myocardium. (a) A typical transverse section shows that most transduced myocytes are found in the left ventricle (inferior wall and part of the lateral wall) and the interventricular septum. The right ventricle is only partly transfected. (b) A longitudinal section confirms the transfected area extending from the basis to the apex. (c) Microscopic analysis demonstrates that X-gal staining concerns essentially cardiac myocytes, even though staining is not homogeneous (magnification ×100).

dium around the vasa vasorum.[19] However, such treatment still remains inefficient.

We used a different strategy and reasoned that a loosening of the pericardial barrier might allow the virus to spread into the myocardium. To achieve this, we tested the possibility of interfering with some of the molecules which constitute this barrier. Among the various components of the extracellular matrix which composes the cardiac interstitium, molecules of collagen type I and type III are important. Thus, as a first step, we tested whether increasing concentrations of collagenase could improve diffusion of the recombinant adenovirus following injection in the pericardium. High doses of collagenase (over 2 mg) appeared to be lethal after a few days and we established that the maximal tolerable dose had to be less than 2 mg for an adult rat. However, under these conditions, transfection of cardiac myocytes remained poor and still limited to the immediate layer underlying the pericardium (data not shown).

Another major component of the extracellular matrix is hyaluronic acid and so we tested the effect of injecting increasing concentrations of hyaluronidase ranging from 1 to 700 units. Even at the highest doses, this enzyme was totally inefficient in facilitating gene transfer to the myocardium across the pericardial barrier (data not shown).

However, when both enzymes were used in conjunction, a different picture emerged. As shown in Figure 2, optimized concentrations of collagenase (1 mg) and hyaluronidase (500 U), when injected together with the recombinant adenovirus allow the virus to diffuse in the myocardium. In particular, a longitudinal section of the heart (Figure 2(b)) clearly indicates that β-galactosidase expression is distributed from the base down to the apex. There is, however, a restriction in the distribution of the expressed gene since transfection mostly concerned the anterior wall of the left ventricle, the interventricular septum, part of the lateral wall of the left ventricle and part

of the right ventricle (Figure 2a). Figure 2c clearly indicates that the cardiac myocytes express the reporter gene. It is also noteworthy that no inflammatory reaction could be found in the transfected area at the time of death.

For comparison, the subdiaphragmatic approach was used to perform direct intracardiac injection. Injection volume had to be adjusted to a maximum volume of 100 µl since injections of larger volumes induce cardiac arrest. Animals were killed at day 7 after injection and transverse cryosections of the heart were assayed for β-galactosidase activity. X-gal staining was measured in order to quantify the transfection area. The adenovirus-transduced cardiac tissue area ranged from 1.4 to 3.9% of the total section. Figure 3 demonstrates the limited extent of the transfection area after direct injection into the left ventricular wall. These results are in close agreement with previously published data by other authors.[9,20]

To validate further the harmlessness of our injection procedure, a number of measurements were made. First, at the time of death, virus-injected animals had similar body weights when compared with control animals injected with identical enzyme solutions but lacking the adenovirus, and with untreated animals. Second, the heart weight/body weight ratios did not differ in any significant way between rats injected with recombinant adenovirus (2.94 ± 0.85 mg/g, $n = 15$) and sham operated animals (2.81 ± 0.41 mg/g, $n = 5$) when killed at 7 days after injection. Lastly, global heart function was analyzed by means of *in vivo* echocardiographic analysis of the left ventricular end diastolic and end systolic dimensions and fractional shortening. A global left ventricular ejection fraction was also calculated. As shown in Table 1, no statistical difference could be found between the various groups of animals, thus confirming the good clinical tolerance achieved by this local method of gene delivery.

To optimize our injection protocol, we tested the efficiency of increasing doses of recombinant adenovirus ranging from 8×10^6 plaque forming unit (p.f.u.) to 4×10^{10} p.f.u. As shown in Table 2, the maximal efficiency of transduction was obtained with 8×10^9 p.f.u. Higher doses did not improve efficiency and were usually found to lead to premature death of the animals (data not shown). All further experiments were conducted using 8×10^9 p.f.u. We also determined the volume of virus which had to be used and found that the best results were obtained with an injection volume of 750 µl. Above this volume, leakage outside the pericardium seems to dominate while below 500 µl, diffusion of the vector around the heart is limited, thus resulting in a smaller area of transduction of the myocardium.

Table 1 Echocardiographic analysis of the left ventricular size and global function.

	Sham	*D7*	*D28*	*P*
n	3	3	2	
LVEDD (mm)	6.6 ± 0.20	5.8 ± 0.0	6.7 ± 0.8	NS
LV EDS (mm)	1.8 ± 0.33	1.6 ± 0.1	2.2 ± 0.6	NS
FS* (%)	73 ± 5%	72 ± 2%	68 ± 5%	NS

*FS (%) = [(LVEDD − LVED)/LVEDD] × 100
Left ventricular end diastolic (LVEDD) and end systolic (LVEDS) dimensions and fractional shortening (FS) were obtained using two-dimensional targeted M-mode tracings (just below the tips of the mitral valve leaflets on a long axis view of the left ventricle) with a 13–15 MHz transducer (Sequoa, ACUSON, Mountain Valley, CA, USA). Three groups of animals were analyzed and all results are expressed as mean ± standard error. No significant modifications were observed in both treated groups compared with sham-operated rats. Sham-operated (Sham) animals received the complete permeabilizing mix without vector and were examined at day 7 after injection. Virus-injected animals were analyzed either at day 7 (D7) or at day 28 (D28).

Figure 3 Direct intracardiac injection by a subdiaphragmatic approach obtains only limited transfection. A transverse section of the ventricles shows the extent of adenovirus-mediated transfection after direct injection of 100 µl of adenoviral vector (8×10^9 p.f.u.) in the presence of the enzymatic mix. Animals were killed at day 7 after injection and β-galactosidase enzymatic activity was revealed by X-gal staining. The diffusion area ranged from 1.4 to 3.9% (magnification ×40).

Table 2 Dose-dependent effect of gene transfer into the rat heart

	n	Transfected area %	P
Control	4	0.00 ± 0.00	
8.0×10^{8}	6	4.18 ± 2.28	0.0083
2.6×10^{9}	4	8.99 ± 3.73	NS
4.0×10^{9}	4	17.18 ± 1.80	0.0209
8.0×10^{9}	8	41.10 ± 6.62	0.0066

The influence of the total viral load was estimated by measuring the X-gal-stained area on transverse sections. Animals were killed at 6.5 days after injection. This planimetric methods which integrates adjacent areas may underestimate sparsely transfected myocardial cells, but is almost operator independent on successive sections. Results are expressed as mean ± standard error and were compared pairwise by statistical tools.

Table 3 Time course of transgene expression in the rat heart

	n	Transfected area %	P
Control	4	0.00 ± 0.00	
D1	4	16.37 ± 13.70	0.0472
D7	8	40.81 ± 9.08	0.0108
D14	4	22.54 ± 7.83	0.0174
D21	4	6.35 ± 1.12	0.0209
D28	4	0.57 ± 0.21	0.0209
D42	4	0.40 ± 0.56	NS

Gene expression in the myocardium was followed from day 1 to day 42 after injection. X-gal-stained areas were expressed as a percentage of the total transverse section area. Results are expressed as mean ± standard error. The evolution of this value was analyzed pairwise by statistical tools.

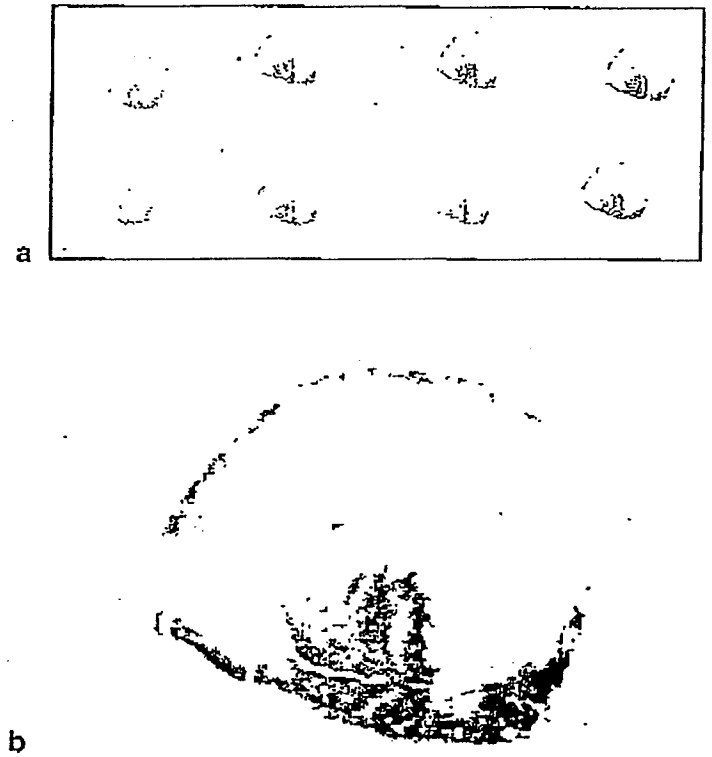


Figure 4 Intrapericardial injections may be achieved in mice. C57BL/6 mice were injected locally with 1×10^{9} p.f.u. of recombinant adenovirus. (a) Serial cryosections of mouse heart demonstrate gene transfer into the heart. (b) A detailed view shows the staining pattern of galactosidase activity after adenovirus-induced transfection. X-gal staining is found in epicardial layers on the cross-section of the mouse heart. Larger areas of transfection can be seen on the interventricular septum and anterior wall of the left ventricle.

A time-course analysis of transgene expression shows that, already at day 1, a significant expression of the transgene can be detected which increases until day 7 when 40.8 ± 7.3% ($n = 8$) of the myocardium is transfected by the adenovirus (Table 3). Thereafter, transgene expression progressively declined due to a significant inflammatory reaction which was found around all areas expressing the transgene (data not shown). This time-course correlates well with previous results reported on direct injection of adenoviral vectors into the myocardium, where transgene expression strongly increased during the first 7 days and then progressively declined at 14 and 21 days.[4] We did not try to immunosuppress our animals since our goal was not to look for long-term gene expression, but to establish the conditions which would allow an optimal viral transduction of the myocardium. It is well known that first generation adenoviral vectors induce an immune response that is a major cause

of transient gene expression in immunocompetent animals and avoidance of an immune response may extend expression of genes introduced into the myocardium.[21,22]

Tissues other than the myocardium were routinely assayed for β-galactosidase activity. As anticipated, lung, liver, spleen and kidneys were always positive; however, the diaphragm muscle never showed evidence of transduction by the adenovirus, indicating that our transdiaphramatic injection did not induce any transfection of the diaphragm.

Our strategy is not limited to the rat, and Figure 4 shows that a comparable result can be obtained with C57BL/6 mice. The distribution of expression is similar to that found in the rat, especially with regard to the regions of the myocardium which are excluded such as the left ventricle (compare Figure 2 and Figure 4).

In conclusion, our results demonstrate that intra-pericardial injection of recombinant adenovirus vectors in the presence of proteolytic enzymes leads to an efficient and safe strategy to deliver a transgene to the heart. No damage to the global heart function could be detected by echocardiographic measurements nor could we find any significant structural alterations of the myocardium at this early stage. As anticipated, we subsequently observed an inflammatory reaction due to the immunogenic potential of both the adenovirus vector and the reporter gene. For future development of this strategy it is obvious that other vectors will need to be used which will include better tailored defective adenovirus or less immunogenic viruses such as the adeno-associated virus[23,24] or even naked DNA. Moreover, the fact that other noncardiac tissues were also efficiently transduced emphasizes the need for another level of control. This will be achieved by placing the therapeutic gene under the control of a cardiac-specific promoter. Finally, the fact that we could transpose our method to another animal model and obtain similar results prompts us to suggest that this approach will be useful for future gene therapy in heart disease.

Acknowledgements

We thank the Vector Core of the University Hospital of Nantes supported by the Association Française contre les Myopathies (AFM) for providing the AdCMVlacZ vector.

References

1 Lin H *et al.* Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA. *Circulation* 1990; 82: 2217–2221.
2 Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J Clin Invest* 1992; 90: 626–630.
3 Von Harsdorf R *et al.* Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals. *Circ Res* 1993; 72: 688–695.
4 French B, Mazur W, Geske R, Bolli R. Direct *in vivo* gene transfer into porcine myocardium using replication-deficient adenoviral vectors. *Circulation* 1994; 90: 2414–2424.
5 Lee J *et al.* Cardiac gene transfer by intracoronary infusion of adenovirus vector-mediated reporter gene in the transplanted mouse heart. *J Thorac Cardiovasc Surg* 1996; 111: 246–252.
6 Coffin RS *et al.* Gene therapy to the heart *in vivo* and to cardiac myocytes and vascular smooth muscle cells *in vitro* using herpes virus vectors. *Gene Therapy* 1996; 3: 560–568.
7 Kypson AP *et al.* *Ex vivo* adenovirus-mediated gene transfer to the adult rat heart. *J Thorac Cardiovasc Surg* 1998; 115: 623–630.
8 Magovern CJ *et al.* Direct *in vivo* gene transfer to canine myocardium using a replication-deficient adenovirus vector. *Ann Thorac Surg* 1996; 62: 425–434.
9 Guzman R *et al.* Efficient gene transfer into the myocardium by direct injection of adenovirus vectors. *Circ Res* 1993; 73: 1202–1207.
10 Barr E *et al.* Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. *Gene Therapy* 1994; 1: 51–58
11 Budker V *et al.* The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Therapy* 1998; 5: 272–276.
12 Donahue JK *et al.* Ultrarapid, highly efficient viral gene transfer to the heart. *Proc Natl Acad Sci USA* 1997; 94: 4664–4668.
13 Wright M *et al.* β-Galactosidase staining following intracoronary infusion of cationic liposomes in the *in vivo* rabbit heart is produced by microinfarction rather than effective gene transfer: a cautionary tale. *Gene Therapy* 1998; 5: 301–308.
14 Davidson BL *et al.* A model system for *in vivo* gene transfer into the central nervous system using an adenoviral vector. *Nat Genet* 1993; 3: 219–223.
15 Lamping K *et al.* Intrapericardial administration of adenovirus for gene transfer. *Am J Physiol* 1997; 272: H310–H317.
16 Lim CS *et al.* Direct *in vivo* gene transfer into the coronary and peripheral vasculatures of the intact dog. *Circulation* 1991; 83: 2007–2011.
17 Muhlhauser J *et al.* Safety and efficacy of *in vivo* gene transfer into the porcine heart with replication-deficient, recombinant adenovirus vectors. *Gene Therapy* 1996; 3: 145–153.
18 Rothmann T *et al.* Heart muscle-specific gene expression using replication defective recombinant adenovirus. *Gene Therapy* 1996; 3: 919–926.
19 Aoki M *et al.* Efficient *in vivo* gene transfer into the heart in the rat myocardial infarction model using the HVJ (hemagglutinating virus of Japan)-liposome method. *J Mol Cell Cardiol* 1997; 29: 949–959.
20 Kass-Eisler A *et al.* Quantitative determination of adenovirus-mediated gene delivery to rat cardiac myocytes *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 1993; 90: 11498–11502.
21 Gilgenkrantz H *et al.* Transient expression of genes transferred *in vivo* into heart using first-generation adenoviral vectors: role of the immune response. *Hum Gene Ther* 1995; 6: 1265–1274.
22 Quinones M *et al.* Avoidance of immune response prolongs expression of genes delivered to the adult rat myocardium by replication-defective adenovirus. *Circulation* 1996; 94: 1394–1401.
23 Lynch CM *et al.* Adeno-associated virus vectors for vascular gene delivery. *Circ Res* 1997; 80: 497–505.
24 Fisher KJ *et al.* Recombinant adeno-associated virus for muscle directed gene therapy. *Nature Med* 1997; 3: 306–312.

EXHIBIT L

Brief Rapid Communication

Expression of Recombinant Genes in Myocardium In Vivo After Direct Injection of DNA

Hua Lin, MD, Michael S. Parmacek, MD, Gerald Morle, BS,
Steven Bolling, MD, and Jeffrey M. Leiden, MD, PhD

The ability to program recombinant gene expression in cardiac myocytes in vivo holds promise for the treatment of many inherited and acquired cardiovascular diseases. In this report, we demonstrate that a recombinant β-galactosidase gene under the control of the Rous sarcoma virus promoter can be introduced into and expressed in adult rat cardiac myocytes in vivo by the injection of purified plasmid DNA directly into the left ventricular wall. Cardiac myocytes expressing recombinant β-galactosidase were detected histochemically in rat hearts for at least 4 weeks after injection of the β-galactosidase gene. These results demonstrate the potential of this method of somatic gene therapy for the treatment of cardiovascular disease. (*Circulation* 1990;82:2217–2221)

Somatic gene therapy, the expression of recombinant genes in non–germ-line tissues of the adult organism, holds great promise for the treatment of many inherited and acquired human diseases (reviewed in Reference 1). The biological requirements for this type of gene therapy include the ability to introduce recombinant genes efficiently into the appropriate cells and tissues and to program the high-level and, in many cases, stable expression of these recombinant genes in vivo. In addition, it is necessary that the process of gene therapy itself not be harmful to the recipient organism, in particular, that the techniques used to introduce the recombinant genes do not result in persistent infection of the host or in deleterious mutations of the recipient cells. Two general approaches have proven useful in animal models of somatic gene therapy. In the first, recombinant genes have been introduced into cultured cells in vitro, and cells expressing the recombinant gene product have then been transplanted into the appropriate tissue of a recipient animal.[2–4] In the second, recombinant genes have been introduced directly into somatic cells in vivo.[5]

From the Howard Hughes Medical Institute (G.M., J.M.L.) and Departments of Internal Medicine (M.S.P., J.M.L.) and Surgery (H.L., S.B.), University of Michigan Medical Center, and the Ann Arbor Veterans Administration Medical Center (M.S.P.), Ann Arbor, Mich.

Supported in part by National Institutes of Health grant DK-42718.

Address for correspondence: Jeffrey M. Leiden, MD, PhD, Howard Hughes Medical Institute, 1150 W. Medical Center Drive, MSRB I, Room 4510, Ann Arbor, MI 48109-0650.

The ability to program recombinant gene expression in adult myocardium in vivo requires both an expression vector with high-level activity in cardiac myocytes and a method for introducing such a vector into myocardial cells in the adult animal. A previous study demonstrated that murine skeletal myocytes display a rather unique ability to take up and express DNA after direct injection in vivo.[6] In the studies described in this report, we show that an expression vector using the Rous sarcoma virus (RSV) long terminal repeat (LTR) programs high-level recombinant gene expression in rat cardiac myocytes in vitro and demonstrate that recombinant genes cloned into this vector can be introduced into and expressed in adult rat cardiac myocytes for at least 4 weeks after direct injection of plasmid DNA into the left ventricular wall.

Methods

Cell Culture and Transient Transfections

Neonatal rat cardiac myocytes were isolated from 1–2-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) by collagenase digestion as previously described.[7] This method results in the isolation of more than 90% cardiac myocytes.[7] Twenty-four hours after isolation, 1×10^6 freshly isolated myocytes in a 60-mm collagen-coated dish (Collaborative Research Inc., Waltham, Mass.) were transfected with 15 μg of cesium chloride gradient–purified chloramphenicol acetyl transferase (CAT) reporter plasmid DNA plus 5 μg of pMSVβgal reference plasmid DNA as follows: 20 μg of plasmid DNA was resuspended in 1.5 ml of Opti-MEM (GIBCO, Grand Island, N.Y.)

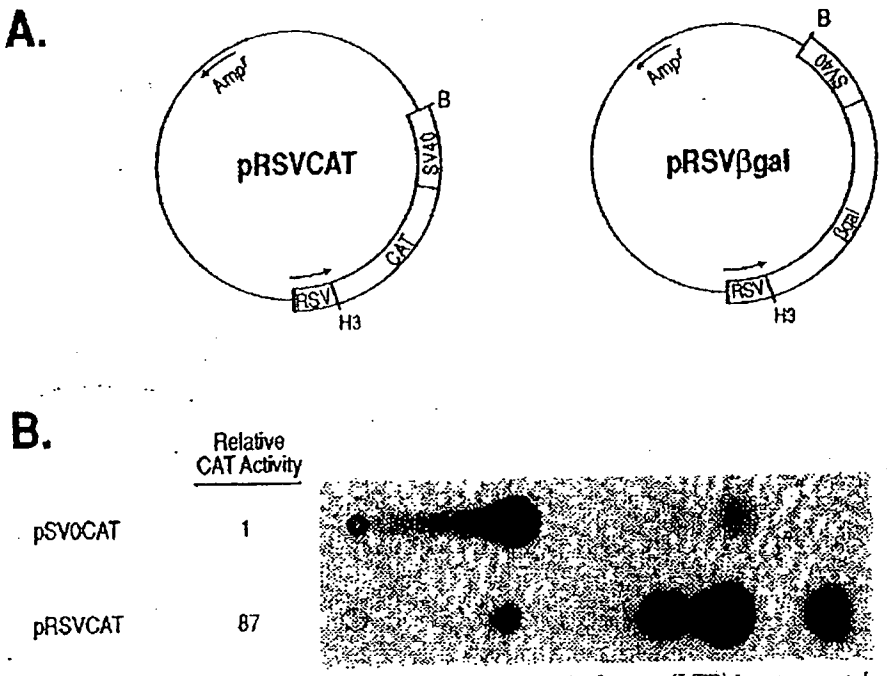


FIGURE 1. *Transcriptional activity of the Rous sarcoma virus (RSV) long terminal repeat (LTR) in rat neonatal cardiocytes in vitro.* Panel A: *A schematic representation of the pRSVCAT and pRSVβgal plasmids. βgal, β-galactosidase gene; CAT, chloramphenicol acetyl transferase gene.* HindIII *(H3) and* BamHI *(B) restriction endonuclease sites are shown.* Panel B: *Transcriptional activity of the RSV LTR in rat neonatal cardiocytes in vitro. Rat neonatal cardiocytes were transfected with 15 μg of the promoterless pSVOCAT control plasmid or the pRSVCAT plasmid (see panel A) and cell extracts prepared 48 hours after transfection were normalized for protein content and assayed for CAT activity as previously described.[9] To control for differences in transfection efficiencies, all transfections also contained 5 μg of the pMSVβgal reference plasmid. Data are shown as CAT activity relative to that produced by the pSVOCAT plasmid (which produced 1.7% acetylation) after correction for differences in transfection efficiency.*

and added to 1.5 ml of Opti-MEM containing 50 μl of lipofectin reagent (BRL, Gaithersburg, Md.). The resulting mixture was added to one 60-mm plate of cardiac myocytes. After 5 hours at 37° C in 5% CO_2, 3 ml of Medium 199 plus 5% fetal bovine serum (FCS) (GIBCO) was added to the cells, and the mixture was incubated at 37° C for 48 hours. Cell extracts were prepared and normalized for protein content using a commercially available kit (Biorad, Richmond, Calif.). CAT and β-galactosidase assays were performed as previously described.[9]

Plasmids

The promoterless pSVOCAT plasmid[10] and the pRSVCAT[11] plasmid in which transcription of the bacterial CAT gene is under the control of the RSV promoter have been described previously. The pRSVβgal plasmid was constructed by cloning the 4.0-kb β-galactosidase gene from pMSVβgal[12] into *Hin*dIII/*Bam*HI-digested pRSVCAT (see Figure 1A).

Injection of Recombinant DNA In Vivo

Six- to 11-week-old 250-g Sprague-Dawley rats were housed and cared for according to National Institutes of Health guidelines in the ULAM facility of the University of Michigan Medical Center. Rats were anesthetized with 20 mg/kg pentobarbitol i.p. and 60 mg/kg ketamine i.m., intubated, and ventilated with a Harvard (Harvard Apparatus, South Natick, Mass.) respirator. A left lateral thoracotomy was performed to expose the beating heart, and 100 μg of plasmid DNA in 100 μl of phosphate-buffered saline (PBS) containing 5% sucrose (PBS/sucrose) was injected into the apical portion of the beating left ventricle using a 30-g needle. Control animals were injected with 100 μl of PBS/sucrose alone. The animals were killed 3–5 or 21–30 days after injection by pentobarbitol euthanasia; hearts were removed via a median sternotomy, rinsed in ice-cold PBS, and processed for β-galactosidase activity.

Histochemical Analysis

Three-millimeter cross sections of the left ventricle were fixed for 5 minutes at room temperature with 1.25% glutaraldehyde in PBS, washed three times at room temperature in PBS, and stained for β-galactosidase activity with X-gal (Biorad) for 4–16 hours as described by Nabel et al.[2] The 3-mm sections were embedded with glycomethocrylate, and 4–7-μm sections were cut and counterstained with hematoxylin and eosin as described previously.[2] Photomicroscopy was performed using Kodak Ektachrome 200 film and Leitz Laborlux D and Wild M8 microscopes.

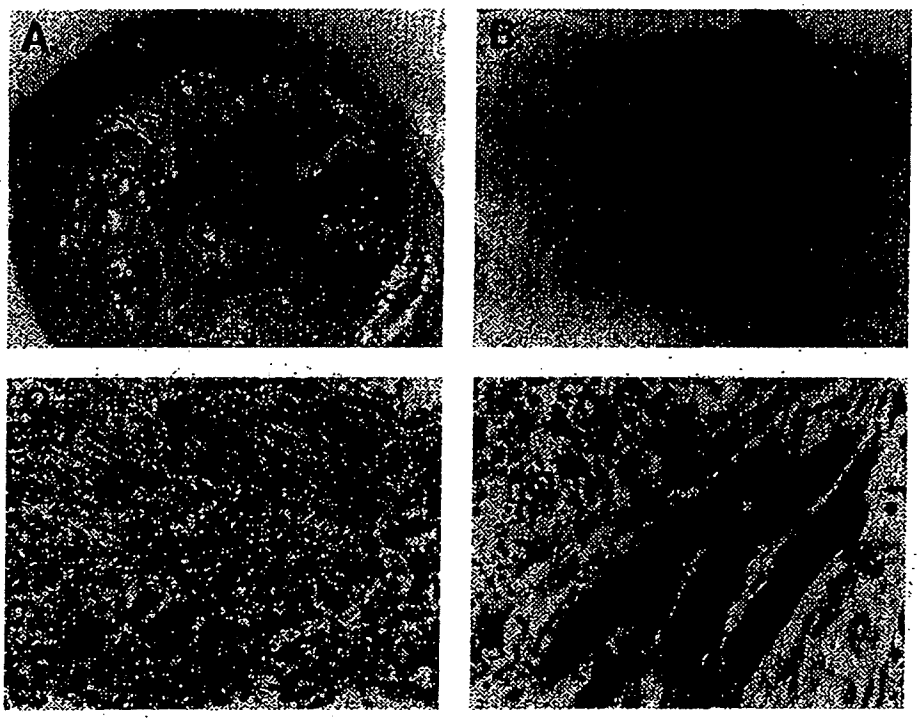


FIGURE 2. *Expression of a recombinant β-galactosidase gene in cardiac myocytes in vivo after direct injection of pRSVβgal DNA into the left ventricular wall. One hundred micrograms of pRSVβgal DNA was injected into the beating apical wall of the left ventricle of Sprague-Dawley rats using a 30-g needle as described in "Methods." Hearts were harvested 3–5 days or 3–4 weeks after injection and stained for β-galactosidase activity.* Panel A: *10× view of a 3-mm section of a heart 3 days after pRSVβgal injection.* Panel B: *18× view of a 3-mm section from a heart 27 days after pRSVβgal injection.* Panels C *and* D: *125× and 250× views, respectively, of 4-μM sections from a heart 3 days after pRSVβgal injection. β-Galactosidase activity (dark-blue staining) is seen only within cardiac myocytes that can be identified by their myofibrillar architecture.*

Results

RSV LTR Promotes High-Level Gene Expression in Rat Neonatal Cardiocytes In Vitro

Although the RSV LTR displays high-level transcriptional promoter activity in a wide variety of immortalized cell types,[11] previous transgenic studies have suggested that this promoter is preferentially active in skeletal and cardiac myocytes in vivo.[13,14] To test directly the transcriptional activity of the RSV LTR in rodent cardiac myocytes, the pRSVCAT vector[11] in which expression of the bacterial CAT gene is under the control of the RSV LTR was transfected into primary neonatal rat cardiac myocytes using lipofectin. Two days after transfection, the cultures were harvested and assayed for CAT activity as previously described.[9] All transfections also contained 5 μg of the pMSVβgal plasmid[12] to correct for differences in transfection efficiencies. As shown in Figure 1, the RSV LTR was able to increase transcription of the CAT gene 87-fold compared with the promoterless pSVOCAT control plasmid. The pRSVCAT-transfected cardiac myocyte extracts produced 95% acetylation in a standard thin-layer chromatography assay.[9] By comparison, identically prepared extracts of 3T3 or HeLa cells transfected with this same vector produced 22% and 35% acetylation, respectively (data not shown). Because the activities of cotransfected pMSVβgal reference plasmids were almost identical in all three transfections, these results demonstrated that the RSV LTR programs high-level transcription in primary cardiac myocytes in vitro.

The ability to unambiguously identify the cell types that are expressing recombinant gene products is an important requirement of all animal models of gene therapy. Because the bacterial β-galactosidase reporter gene (but not the bacterial CAT gene) allows direct histological visualization of recombinant gene expression, we constructed a pRSVβgal vector in which bacterial β-galactosidase gene expression is regulated by the RSV LTR promoter for further studies of recombinant gene expression in vivo (Figure 1B).

Expression of β-Galactosidase Gene in Rat Cardiac Myocytes After Injection of pRSVβgal DNA Into the Left Ventricular Wall In Vivo

In an attempt to program recombinant β-galactosidase gene expression in rat cardiac myocytes in

vivo, we took advantage of a previously described technique for producing recombinant gene expression in murine skeletal myocytes in vivo.[6] Briefly, 100 μg of pRSVβgal DNA was resuspended in 100 μl of PBS containing 5% sucrose (PBS/sucrose) and injected via a 30-g needle directly into the beating left ventricular wall of 6–11-week-old Sprague-Dawley rat hearts. Control rats received injections of 100 μl of PBS/sucrose without DNA. Rats were killed either 3–5 days or 3–4 weeks after injection, and hearts were fixed and stained for β-galactosidase activity. β-Galactosidase activity as manifested by dark-blue staining was readily apparent to the naked eye in sections of three of four of the pRSVβgal-injected hearts at 3–5 days and four of five of the pRSVβgal-injected hearts at 3–4 weeks after DNA injections (Figures 2A and 2B). This staining, which was focal and patchy, occurred only in a single area of each heart injected with pRSVβgal DNA and was not seen in five control hearts injected with PBS/saline alone (data not shown). Failure to observe staining in two of nine of the pRSVβgal-injected hearts may have been due to the lack of DNA uptake or expression in these hearts or, more likely, to technical difficulties in successfully centering and anchoring the needle in the relatively thin beating left ventricular wall during the injection process.

Because the normal ventricular wall contains both myocytes and fibroblasts and because the injection of DNA might be expected to cause a localized inflammatory response, it was important to determine which cell types were expressing the recombinant β-galactosidase gene. Histochemical analysis of sections from hearts injected with the pRSVβgal DNA clearly demonstrated β-galactosidase activity within cardiac myocytes that were easily identified by their myofibrillar architecture (Figures 2C and 2D). Between one and 10 positively staining myocytes were seen per high-power field, and these were often noncontiguous, suggesting that the uptake of DNA and/or its expression is a relatively low-frequency event. Because it was difficult to accurately identify the extent of DNA injection and because the positively staining areas were quite focal and patchy, it was impossible to accurately quantitate either the percentage or the total number of cells expressing recombinant β-galactosidase activity in a given heart. However, it is clear that only a small fraction of cardiac myocytes expressed the recombinant protein. In addition, it is worth noting that sections from the 3–5-day postinjection hearts often showed evidence of an acute inflammatory response along the track of the needle (Figure 2C) and that in several cases fibrosis along the needle track was observed in sections from the 3–4-week postinjection hearts (data not shown).

Discussion

The studies presented have demonstrated that it is possible to program recombinant gene expression in cardiac myocytes after direct injection of DNA into the left ventricular wall. Functional recombinant protein expression in myocytes was demonstrated directly using an enzymatic assay for β-galactosidase. Recombinant gene expression was observed in myocytes from seven of nine of the injected hearts at both 3–5 days and 3–4 weeks after injection. Expression was patchy and was observed only in direct contiguity with the site of injection. These findings have several implications regarding both the use of this method for somatic gene therapy in the heart and the biology of recombinant DNA uptake and expression in muscle cells.

A previous study suggested that murine skeletal muscle cells possess a unique ability to take up and express injected recombinant DNA.[6] Our results have extended this observation to cardiac muscle cells in a second rodent species. It has previously been thought that successful DNA transfection and expression may require recipient cell division and, more specifically, breakdown of the recipient cell nuclear membrane to allow DNA entry. Because skeletal myocytes have a limited potential for mitosis,[15] it remained possible that the previously reported successful transfection of skeletal myocytes was dependent on their mitotic potential. In contrast to skeletal myocytes, adult cardiac muscle cells are unable to divide.[16] Thus, our results demonstrate that mitosis is not necessary for successful transfection of cells with DNA. The mechanisms that allow preferential uptake of DNA into cardiac and skeletal myocytes remain unclear. However, our data suggest that they must be dependent on structural or functional properties that are shared by skeletal and cardiac muscle. Current hypotheses include the possibility of specialized muscle cell transport systems or the unique ability to physically disrupt the cell membranes of muscle cells in a reversible fashion during the recombinant DNA injections.

The technique of somatic gene therapy using direct DNA injection into myocardium, as described in this report, has several advantages compared with other previously described methods of gene therapy. First, infectious viral vectors are not required, eliminating the possibility of persistent infection of the host. Second, a previous study[6] has suggested that recombinant DNA taken up and expressed in skeletal myocytes persists as an episome and therefore does not have the same potential for host cell mutagenesis as do retroviral vectors that integrate into the host chromosome. Finally, this method does not require the growth of recipient cells in vitro, a requirement that would render transfection of nondividing cardiac myocytes particularly difficult.

Direct injection of recombinant DNA into the myocardium holds promise for the treatment of many acquired and inherited cardiovascular diseases. We are particularly interested in the possibility of stimulating collateral circulation in areas of chronic myocardial ischemia by expressing recombinant angiogenesis factors locally in the ventricular wall. Although the method described in this report is a first step toward such gene therapy approaches, many questions and problems remain to be addressed

before this type of gene therapy can become a reality. First, it must be demonstrated that human myocytes, like their rodent counterparts, are able to take up and express recombinant DNA. The longevity of recombinant gene expression must be more fully examined, and the possibility that some of the recombinant DNA is integrated into the host genome with the concomitant potential for mutagenesis must be ruled out. Modifications of the current transfection protocol must be developed to increase the frequency of recombinant gene expression in cardiac myocytes. Of equal importance, the inflammatory response to the injected DNA must be controlled to prevent the formation of arrhythmogenic foci. Finally, it will of interest to determine whether high-level recombinant gene expression can be programmed in vivo by the injection of expression vectors containing cardiac-specific transcriptional regulatory elements. Ongoing studies in our laboratory are designed to address these problems. Nevertheless, the initial studies described in this report suggest that somatic gene therapy in the heart may eventually become a useful therapeutic modality.

Acknowledgments

The authors would like to thank Dr. James Wilson for critical review of the manuscript, Mrs. Jeanelle Pickett for expert secretarial assistance, and Ms. Beverly Burck for the preparation of the illustrations. We would also like to thank Kaye Brabec, Lorita Dudus, and Dr. Kent Christensen for help with histochemical staining.

References

1. Swain JL: Gene therapy: A new approach to the treatment of cardiovascular disease. *Circulation* 1989;80:1495–1496
2. Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ: Recombinant gene expression *in vivo* within endothelial cells of the arterial wall. *Science* 1989;244:1342–1344
3. Wilson JM, Birinyi LK, Salomon RN, Libby P, Callow AD, Mulligan RC: Implantation of vascular grafts lined with genetically modified endothelial cells. *Science* 1989;244:1344–1346
4. Dichek DA, Neville RF, Zwiebel JA, Freeman SM, Leon MB, Anderson WF: Seeding of intravascular stents with genetically engineered endothelial cells. *Circulation* 1989;80:1347–1353
5. Nabel EG, Plautz G, Nabel GJ: Site-specific gene expression in vivo by direct gene transfer into the arterial wall. *Science* 1990 (in press)
6. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL: Direct gene transfer into mouse muscle in vivo. *Science* 1990;247:1465–1468
7. Engelmann GL, Gerrity RG: Biochemical characterization of neonatal cardiomyocyte development in normotensive and hypertensive rats. *J Mol Cell Cardiol* 1988;20:169–177
8. Curran T, Miller AD, Zokas L, Verma IM: Viral and cellular fos proteins: A comparative analysis. *Cell* 1984;36:259–268
9. Parmacek MS, Bengur AR, Vora AJ, Leiden JM: Structure and regulation of expression of the murine fast skeletal troponin C gene: Identification of a developmentally-regulated, muscle-specific transcriptional enhancer. *J Biol Chem* 1990 (in press)
10. Gorman CM, Moffat LF, Howard BH: Recombinant genomes which express chloramphenicol acetyl-transferase in mammalian cells. *Mol Cell Biol* 1982;77:1432–1436
11. Gorman C, Padmanabhan R, Howard BH: High efficiency DNA-mediated transformation of primate cells. *Science* 1983; 221:551–553
12. Donoghue M, Ernst H, Wentworth B, Nadal-Ginard B, Rosenthal N: A muscle-specific enhancer is located at the 3' end of the myosin light-chain 1/3 gene locus. *Genes Dev* 1988;2:1779–1790
13. Overbeek PA, Lai S-P, Van Quill KR, Westphal H: Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. *Science* 1986;231:1574–1577
14. Swain JL, Stewart TA, Leder P: Parental legacy determines methylation and expression of an autosomal transgene: A molecular mechanism for parental imprinting. *Cell* 1987;50: 719–727
15. Zak R: Development and proliferation capacity of cardiac muscle cells. *Circ Res* 1974;34-35(suppl II):II-17–II-26
16. Korecky B, Rakusan K: Normal and hypertrophic growth of the rat heart: Changes in cell dimensions and numbers. *Am J Physiol* 1978;234:H123–H128

KEY WORDS • gene therapy • DNA

EXHIBIT M

Rapid Publication

Widespread Long-term Gene Transfer to Mouse Skeletal Muscles and Heart

Leslie D. Stratford-Perricaudet,* Iman Makeh,‡ Michel Perricaudet,* and Pascale Briand*
*Institut Gustave Roussy, Centre National de la Recherche Scientifique, UA 1301, 94805 Villejuif, France; and ‡Institut Cochin de Génétique Moléculaire, Institut National de la Santé et de la Recherche Médicale, CJF 9003, 75014 Paris, France

Abstract

Successful treatment of muscular disorders awaits an adapted gene delivery protocol. The clinically applicable technique used for hematopoietic cells which is centered around implantation of retrovirally modified cells may not prove sufficient for a reversal of phenotype when muscle diseases are concerned. We report here efficient, long-term in vivo gene transfer throughout mouse skeletal and cardiac muscles after intravenous administration of a recombinant adenovirus. This simple, direct procedure raises the possibility that muscular degenerative diseases might one day be treatable by gene therapy. (*J. Clin. Invest.* 1992. 90:626–630.) Key words: adenovirus • gene therapy • β-galactosidase • muscular disease • eukaryotic viral vector

Introduction

The first genetic disorders amenable to gene transfer–based treatment will be monofactorial diseases. The vast array of target tissues translates the need for the development of appropriate, efficient gene transfer vehicles. The ability of retroviruses to integrate into the host genome has led to their use in ex vivo treatment protocols. Because those cell types capable of withstanding extraction, in vitro manipulation, and, finally, reimplantation are quite limited, other strategies need to be explored. Furthermore, the requirement retroviruses have for host cell proliferation constitutes an important drawback of such vectors and limits their applicability. Many targets relevant to human disease (liver, lung, muscle, neurons) will require other means of gene transfer.

Efficient and long-term expression of genes adenovirally transduced has recently been reported in hepatocytes and bronchial epithelium of animals (1–3), showing that the adenoviral vector is capable of transferring genes to nondividing or slowly proliferating cells. To investigate other potential targets for recombinant adenoviral vectors, we have constructed a recombinant adenovirus expressing a nuclearly targeted reporter enzyme (Ad.RSVβgal). The rapid in situ detection of the nuclearly targeted β-galactosidase allows an unambiguous and precise appreciation of adenoviral-mediated gene transfer (4, 5).

Address correspondence to Leslie D. Stratford-Perricaudet, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif, France.
Received for publication 13 January 1992 and in revised form 7 April 1992.

J. Clin. Invest.

0021-9738/92/08/0626/05 $2.00
Volume 90, August 1992, 626–630

Methods

Construction of recombinant plasmid pAd.RSVβgal. The pAd.RSVβgal is a pML-2 derivative where the nls lacZ gene with the SV40 early region polyadenylation signal (5, 6) driven by the Rous sarcoma virus long terminal repeat (RSV LTR)[1] is inserted downstream of 1.3 map units (mu) (PvuII site) from the left end of the adenovirus type 5 (Ad5) genome. The reporter gene is followed by mu 9.4–17 (BglII–HindIII fragment) of Ad5 to allow homologous recombination with the adenoviral genome for the generation of the recombinant adenovirus (Fig. 1).

Construction of recombinant adenovirus Ad.RSVβgal. The recombinant adenovirus was constructed by in vivo homologous recombination (7) in 293 cells (8) between plasmid pAd.RSVβgal and Ad dl327 (9) genomic DNA. Briefly, 293 cells were cotransfected with 5 μg of linearized pAd.RSVβgal and 5 μg of the large ClaI fragment (2.6–100 mu) of Ad5 DNA. After overlaying with agar and incubation for 10 d at 37°C, plaques containing recombinant adenovirus were picked and screened for nuclear β-galactosidase activity. The recombinant virus was propagated in 293 cells and purified by cesium chloride density centrifugation. Titers of the viral stocks were determined by plaque assay using 293 cells.

Injection of mice. 2- to 5-d-old and adult (2 mo) mice (C57BL6 × DBA) were injected either intravenously (iv) or intramuscularly (im) (quadriceps) with 20–40 μl of highly purified recombinant adenovirus, Ad.RSVβgal (10^{11} plaque-forming units [pfu]/ml).

Escherichia coli β-galactosidase assay. Organs from killed animals were fixed in 4% p-formaldehyde in PBS for 30 min. After rinsing they were incubated overnight at 30°C in X-gal solution (2 mM) (6). Whole specimens were flash frozen in isopentane in liquid nitrogen and mounted in OCT compound (Miles Laboratories Inc., Naperville, IL) for cryosectioning. Sections (10 μm thick) were fixed 10 min in p-formaldehyde as described for organs, rinsed, and incubated with X-gal substrate. Sections were then counterstained with hematoxylin and eosin according to standard methods. Muscle was dissociated after whole organ staining to obtain isolated myofibers that were then counterstained with hematoxylin and eosin. Urine and fecal matter were collected at 2 h or 22 d after iv injection and exposed to 293 cells. After 24 h of incubation at 37°C the cells were fixed and stained with X-gal solution.

DNA analysis of animals. The heart, lung, liver, and quadricep muscle from mice iv-injected with the recombinant adenovirus Ad.RSVβgal were minced into liquid N_2 and ground with a mortar and pestle. Total cellular DNA was prepared as described (10) and 10 μg of either undigested or HindIII-digested DNA was subjected to electrophoresis in a 0.8% agarose gel. Southern blot analyses (Fig. 4 *A*) were performed using either a SalI-BamHI fragment containing LacZ from pGEM-nlsLacZ (6), or a fragment from the pAd.RSVβgal plasmid containing the RSV promoter and the upstream adenovirus sequences (Fig. 4 *B*) to screen for the presence of the recombinant adenovirus.

1. *Abbreviations used in this paper:* Ad5, adenovirus type 5; DMD, Duchenne muscular dystrophy; mu, map units; pfu, plaque-forming units; RSV LTR, Rous sarcoma virus long terminal repeat.

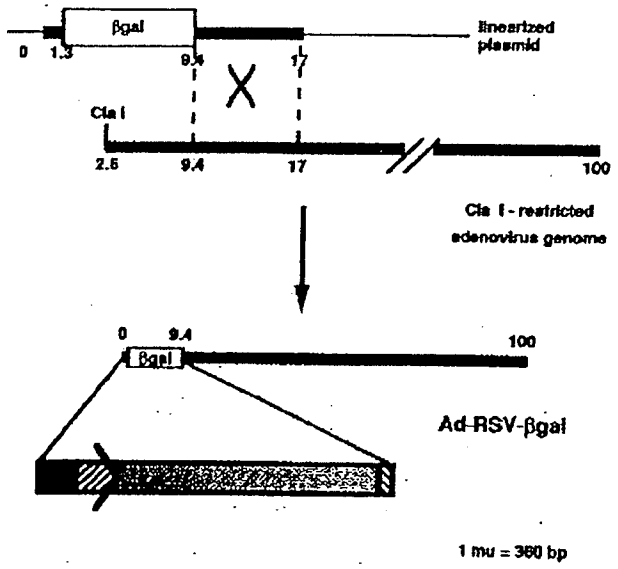


Figure 1. Generation of recombinant adenovirus, Ad.RSVβgal, by in vivo recombination. The recombinant adenovirus was constructed through homologous recombination between plasmid pAd.RSVβgal and the Ad5 genome. Shown also is an enlargement of the insert. The adenoviral sequences are depicted by ■. The nlslacZ gene □ is controlled by the RSV LTR ◘ and possesses the early mRNA polyadenylation signal from SV40 ◘.

Results

Generation of the recombinant adenovirus, Ad.RSVβgal. Ad.RSVβgal is a recombinant adenovirus that constitutively expresses β-galactosidase targeted to the nuclei of infected cells. The nls lacZ coding sequence with the SV40 early region 3'-end processing signal and under the control of the RSV LTR was inserted into Ad dl327 in place of E1a and E1b (mu 1.3–9.4). Fig. 1 depicts the construction of the recombinant virus. Plaques resulting from the transfection of 293 cells were screened for β-galactosidase activity. Virus was amplified on 293 cells. The recombinant virus is replication incompetent due to its deletion for the E1 genes.

Expression of the transferred gene in mice injected as neonates. Neonatal mice were intravenously injected with the Ad.RSVβgal recombinant virus, and gene transfer was assessed by histochemical staining for β-galactosidase activity in various tissues. The extent of blue staining reveals that a substantial percentage of cells within different tissues are infected. Positive perinuclear staining was systematically observed in many organs such as lung, liver, intestine, heart, and skeletal muscle (Fig. 2, *A–F*) of each of the four individual mice killed at 15 d after iv injection.

The exciting implications of efficient gene transfer into myocytes led us to characterize transduction to these cells in particular. Gross examination of the intact heart as well as skeletal muscles from the experimental animals reveals the impressive efficiency of gene transfer after only a single injection of the recombinant adenovirus (Fig. 2, *C, D,* and *F*). Because the intravenous route was used, the viral vector is not concentrated in any one area of the muscle tissue, and dispersion is favored. Histochemical staining of muscle leads to large patches of blue throughout. Approximately 0.2% of cardiac cells have undergone gene transfer after iv injection of 10^9 pfu of virus.

Expression of the transferred gene in both cardiac and skeletal muscle was found to be remarkably stable since monthly killed injected animals displayed β-galactosidase activity in these tissues throughout the 12-mo period of the experiment. Importantly, gene expression was sustained, although the proportion of blue cells in these muscle tissues seemed to decrease after the 10th month after injection.

Analysis of isolated fibers demonstrates the extent of dissemination of the transferred gene. A single fiber can show multiple "centers of expression" (Fig. 3). Each consists of a darkly stained central nucleus surrounded by nuclei forming a gradient of blue. These centers most probably result from independent local infections by the adenoviral vector. The number of colored nuclei in any one cluster was found to vary from 10 to 60. The use of a nuclearly targeted marker is informative as to the degree of infection of multinucleated cells of this morphology.

Expression of the transferred gene in mice injected as adults. As to the distribution of the virus, results similar to those obtained in mice injected as newborns were found after intravenous inoculation of adults. It is apparent, however, that the injection of 10^9 pfu of virus leads to less efficient gene transfer in the larger adult mice. Intramuscular injections were also performed and histochemical staining at 21 days after injection revealed that the infection was circumscribed to the point of injection, as fibers with blue nuclei could only be detected within a 1-cm area. Moreover, the absence of blue staining in other tissues (lung, liver, intestine) reveals a very limited diffusion of the virus when the intramuscular route is chosen.

Status of the viral DNA. Southern blot analysis of DNA from different tissues of an experimental animal indicates the presence of the adenovirus genome in a wide variety of organs (Fig. 4 *A*). The detection of a DNA fragment corresponding to the left end of the recombinant viral genome indicates that the viral DNA is present as a linear form in the tissues infected 10 d previously (Fig. 4 *B*). Moreover, the detection of a unique and intense band corresponding to 35.5 kb at 3 mo after injection reveals that the viral DNA remains extrachromosomal (Fig. 5). The absence of a detectable smear rules out the possibility that integration events occurred with high frequency. Clearly, the sensitivity of the Southern blot does not allow a fair appreciation of gene transfer as does the in situ detection of the β-galactosidase activity. In this respect, it is reasonable that the estimation of a 0.2% transfer to the heart (based on staining) is compatible with the difference in intensity seen with the transgenic mouse DNA control (which harbors one copy of a LacZ gene per cell) (Fig. 4 *A*). The incapacity to detect a band in the heart 12 mo after injection is in agreement with the decrease in the number of blue cells observed at this time.

Discussion

Gene therapy relevant to muscular diseases is especially hindered by the unsolved problem of the direct widespread transfer of a gene to the related tissues. Attempts to modify muscle tissue have centered around fusion of implanted myocytes with host muscle (11, 12) or injection of DNA (13–15). Fusion in mice of normal donor muscle precursor cells with host mdx myofibers (11) has borne excitement leading to preliminary trials of such cell therapy in children. This approach

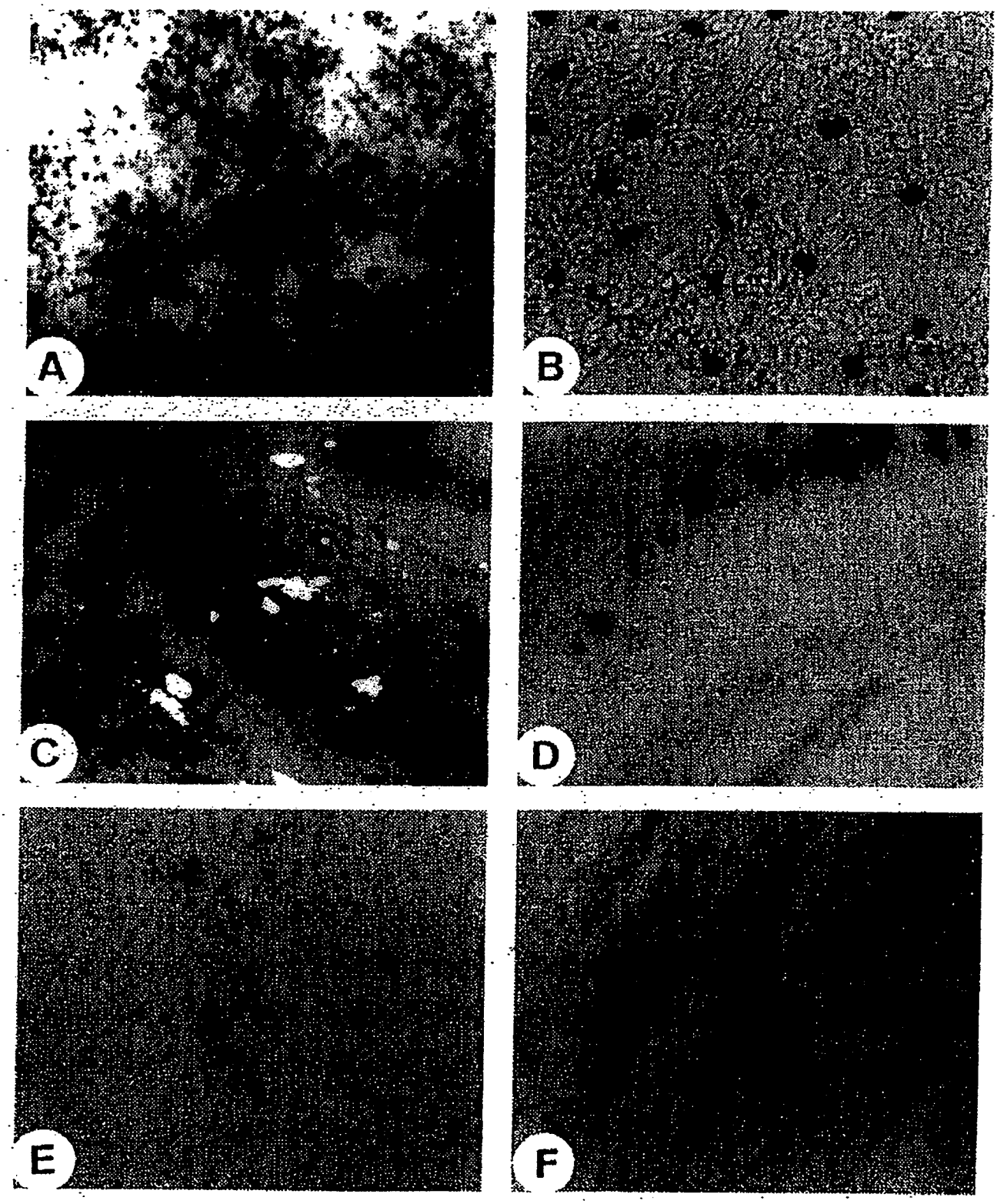


Figure 2. Efficient adenoviral-mediated gene transfer to mouse organs as evidenced by histochemical staining for β-galactosidase activity. (*A*) Liver (×40); (*B*) lung (×400); (*C*) heart atrium (×40); (*D*) heart ventricle (×40); (*E*) intestine (×40); (*F*) skeletal muscle (×40). Newborn mice were injected intravenously and organs were removed 15 d after injection for in situ assay for β-galactosidase activity.

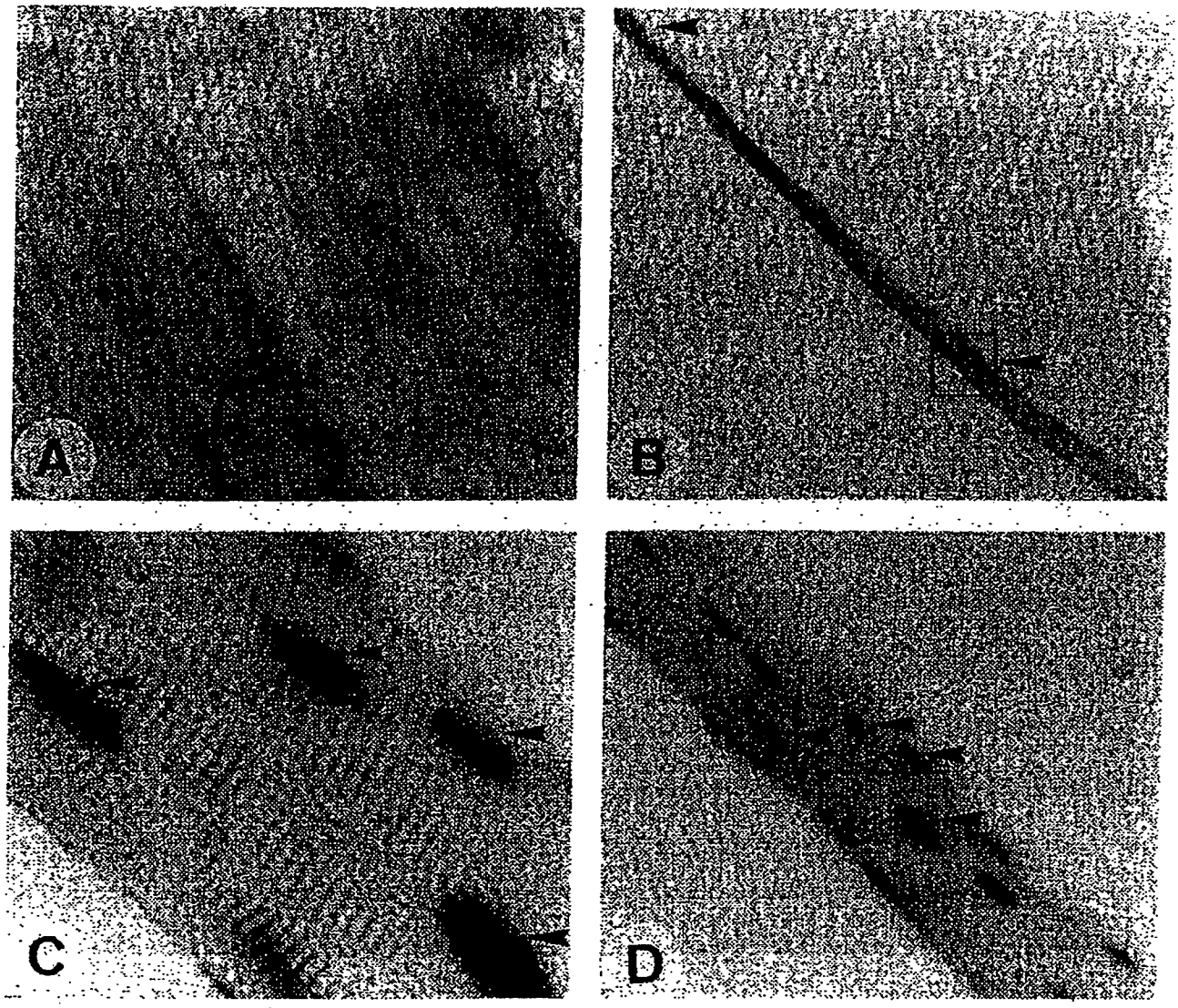


Figure 3. Detailed analysis of gene transfer into skeletal muscle after iv injection of Ad.RSVβgal. Gene expression was assessed 5 mo after injection by histochemical staining for nuclear β-galactosidase activity. (*A*) Dorsal skeletal muscle (×40); (*B*) isolated dorsal muscle fiber (×40). Arrowheads indicate two centers of expression. (*D*) Enlargement of boxed area in *B* (×250). A dark blue source nucleus is surrounded by nuclei of varying intensity of blue staining. (*C*) Enlargement of center of expression shown in *D* (×500). Arrowheads indicate source nucleus and nuclei of immediate vicinity.

may, nevertheless, have too many drawbacks for it to be applicable to the treatment of disease. As the migratory capacities of precursor cells are restricted to a few millimeters, cell implantation would necessitate millions of injections during hours of anesthesia. Inevitably, immunological problems would be encountered, as with any graft. Furthermore, large scale requirements for human myogenic cells constitute a practical limitation. In addition, the treatment of Duchenne muscular dystrophy (DMD) not only calls for therapy for skeletal muscles, but for myocardial cells too. It is difficult to envisage cell therapy as a means to provide relief to such an array of diseased cells.

The concept of somatic gene therapy will more than likely provide the most promising solution in the future. Importantly, its scope goes beyond the treatment of muscle disease, since it is applicable to a large number of genetic disorders. The direct introduction of purified nucleic acids into various organs in vivo is attractive due to its simplicity, but again, practical obstacles may limit its development. Furthermore, the resultant gene expression in muscle remains localized to the point of injection of DNA (13) and seems to be quite limited in duration, particularly in cardiac muscle (14). Interestingly, all other organs tested proved to be nonreceptive to DNA transfection. Thus, a method allowing a more widespread distribution of stable gene expression would be of invaluable importance to gene therapy in general.

The present report demonstrating the feasibility of adenovirus-mediated direct in vivo gene transfer into myocytes of mice has serious implications for treatment of muscular disorders, including heart diseases. The proportion of skeletal and myocardial cells expressing the transferred gene is more in keeping with that probably required for a reversal of disease state. It is

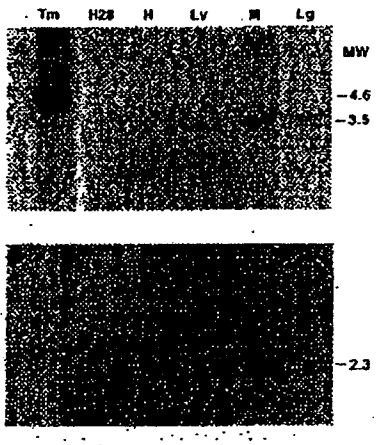


Figure 4. Southern blot analyses. All lanes contain 10 μg of HindIII-digested tissue DNA hybridized either with a Lac Z probe (*A*) or with a probe specific for the left end of the recombinant virus (*B*). Tm corresponds to the DNA of a transgenic mouse which contains one copy/cell of a Lac Z gene. DNA from the heart (*H*), liver (*Lv*), skeletal muscle (*M*), and lung (*Lg*) of a mouse killed at 10 d after injection were analyzed. H28 corresponds to heart DNA from a mouse killed 12 mo pi. Size markers (*MW*) are shown in kilobase-pairs in the right margin.

expected that the percentage of recipient cells be a function of the quantity of injected virus per animal weight. The possibility to obtain very high titers of adenovirus makes it conceivable to increase the input of virus for larger animals. The remarkable stability of expression observed (at least 12 mo), notwithstanding the extrachromosomal state of the vector, would be of safe therapeutic value.

An important distribution of the putative therapeutic DNA is a prerequisite for the treatment of muscular diseases like DMD. This can clearly be achieved when a recombinant adenovirus is administered intravenously since in this case dispersion of the vector occurs throughout the animal. In contrast, the direct intramuscular injection of a recombinant adenovirus can only lead to a localized gene transfer. It is noteworthy that gene transfer is successful not only in neonatal animals, but also in adults (albeit to a lesser extent), thus opening the route to gene therapy of diseases clinically diagnosed later in life.

Importantly, adenovirus can carry tissue-specific promoters, consequently restricting the actual sites of expression of the exogenous gene (16, 17). Moreover, the important cloning capacity of the adenovirus vector makes realistic and promising the construction of a recombinant adenovirus harboring the dystrophin gene. The recent report showing that expression of dystrophin can correct one of the effects of dystrophin deficiency (15), can only stress the urgency of an adapted vector. Taken together, the potentials of adenovirus along with its proven capacities, render this virus a most interesting gene delivery system for the treatment of the important hereditary human disease, DMD. The construction of an adenovirus harboring the dystrophin cDNA is now in progress in the laboratory. If such a virus is to be used in humans, safety aspects should be addressed concerning viral dissemination in the local environment. In this regard, it is noteworthy that no Ad.RSVβgal virus could be detected in urine and fecal matter after intravenous inoculation of the recombinant adenovirus even though a nuclear Lac Z expression could be detected not only in the smooth muscle of the intestine (Fig. 2 *E*), but also occasionally in a few epithelial cells of this organ (data not shown).

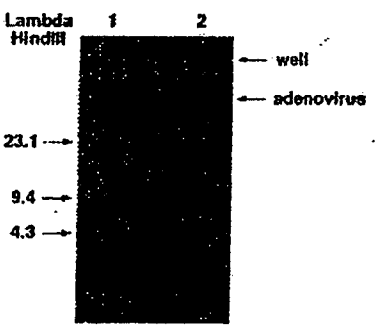


Figure 5. Southern blot analysis. Lane *1*, 10 μg of undigested tissue DNA prepared from a control heart; lane *2*, 10 μg of undigested tissue DNA prepared from the heart of an experimental mouse injected intravenously 3 mo previously. Size markers (Lambda/HindIII) are shown in kilobasepairs in the left margin, and arrows in the right margin indicate loading wells and adenovirus size marker.

Acknowledgments

We thank A. Kahn for a critical discussion and revision of the manuscript. We thank V. Chevalier for typing the manuscript.

Our work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, and the Association Française contre les Myopathies.

References

1. Stratford-Perricaudet, L. D., M. Levrero, J.-F. Chasse, M. Perricaudet, and P. Briand. 1990. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Hum. Gene Ther.* 1:241–256.

2. Rosenfeld, M. A., W. Siegfried, K. Yoshimura, K. Yoneyama, M. Fukayama, L. E. Stier, P. K. Paakko, P. Gilardi, L. D. Stratford-Perricaudet, M. Perricaudet, et al. 1991. Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium in vivo. *Science (Wash. DC).* 252:431–434.

3. Rosenfeld, M. A., K. Yoshimura, B. Trapnell, K. Yoneyama, E. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L. Stier, L. D. Stratford-Perricaudet, et al. 1992. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 68:143–155.

4. Ralston, E., and Z. W. Hall. 1989. Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. *Science (Wash. DC).* 244:1066–1069.

5. Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499–509.

6. Bonnerot, C., D. Rocancourt, P. Briand, G. Grimber, and J.-F. Nicolas. 1987. A β-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. USA.* 84:6795–6799.

7. Gluzman, Y., H. Reichl, and D. Solnick. 1982. Helper-free adenovirus type 5 vectors. *In* Eukaryotic Viral Vectors. Y. Gluzman, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 187–192.

8. Graham, F. L., J. Smiley, W. C. Russel, and R. Nairu. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59–72.

9. Shenk, T., and J. Williams. 1984. Genetic analysis of adenoviruses. *Curr. Top. Microbiol. Immunol.* 111:1–39.

10. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

11. Partridge, T. A., J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel. 1989. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature (Lond.).* 337:176–179.

12. Salminen, A., H. F. Elson, L. A. Mickley, A. T. Fojo, and M. M. Gottesman. 1991. Implantation of recombinant rat myocytes into adult skeletal muscle: a potential gene therapy. *Hum. Gene Ther.* 2:15–26.

13. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. 1991. Direct gene transfer into mouse muscle in vivo. *Science (Wash. DC).* 247:1465–1468.

14. Acsadi, G., S. Jiao, A. Jani, D. Duke, P. Williams, W. Chong, and J. A. Wolff. 1991. Direct gene transfer and expression into rat heart in vivo. *New Biol.* 3:71–81.

15. Acsadi, G., G. Dickson, D. R. Love, A. Jani, F. S. Walsh, A. Gurusinghe, J. A. Wolff, and K. E. Davies. 1991. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. *Nature (Lond.).* 352:815–818.

16. Friedman, J. M., L. E. Babiss, D. F. Clayton, and J. E. Darnell, Jr. 1986. Cellular promoters incorporated into the adenovirus genome: cell specificity of albumin and immunoglobulin expression. *Mol. Cell. Biol.* 6:3791–3797.

17. Karlsson, S., K. van Doren, S. Schweiger, A. Nienhuis, and Y. Gluzman. 1986. Stable gene transfer and tissue-specific expression of a human globin gene using adenoviral vectors. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2377–2385.

EXHIBIT N

Gene Injection Into Canine Myocardium as a Useful Model for Studying Gene Expression in the Heart of Large Mammals

Rüdiger von Harsdorf, Robert J. Schott, You-Tang Shen, Stephen F. Vatner, Vijak Mahdavi, and Bernardo Nadal-Ginard

We have investigated the regulated expression of genes injected into the heart of large mammals in situ. Reporter constructs using the chloramphenicol acetyltransferase gene under the control of muscle-specific β-myosin heavy chain (β-MHC) or promiscuous (mouse sarcoma virus) promoters were injected into the canine myocardium. There was a linear dose–response relation between the level of gene expression and the quantity of plasmid DNA injected between 10 and 200 μg per injection site. The level of reporter gene expression did not correlate with the amount of injury imposed on the cardiac tissue. There was no regional variation in expression of injected reporter genes throughout the left ventricular wall. By use of both the mouse sarcoma virus and a muscle-specific β-MHC promoter, reporter gene expression was one to two orders of magnitude greater in the heart than in skeletal muscle. Expression in the left ventricle was threefold higher than in the right ventricle. Chloramphenicol acetyltransferase activity was detected at 3, 7, 14, and 21 days after injection, with maximal expression at 7 days after injection. Statistical analysis of coinjection experiments revealed that coinjection of a second gene construct (Rous sarcoma virus–luciferase) is useful in the control of transfection efficiency in vivo. Furthermore, using reporter constructs containing serial deletions of the 5′ flanking region of the β-MHC gene, we performed a series of experiments that demonstrate the utility of this model in mapping promoter regions and identifying important regulatory gene sequences in vivo. Thus, gene injection into canine myocardium has proven to be a powerful tool in the study of regulated gene expression in large mammals in vivo, with the potential of providing useful clues about the regulation of gene expression prevailing in human myocardium. (*Circulation Research* 1993;72:688–695)

KEY WORDS • in vivo gene transfer • large mammals • canine myocardium • β-myosin heavy chain • in vivo promoter mapping

Until recently, regulated gene expression in vivo has been studied by using transfection assays of cultured cells[1–5] and/or by creating transgenic animals.[6,7] Particularly in the case of terminally differentiated cells, such as the cardiac myocytes, the use of cultured cells has been hampered by low transfection efficiencies and the difficulty in reproducibly obtaining sufficient quantities of primary cells when phenotypically suitable established cell lines are not available. These problems are further accentuated in the study of genes expressed in the myocardium. Indeed, even in the best of cases, the interpretation and physiological significance of the results is weakened by the fact that the cells are not embedded in their natural environment. These reasons and the paucity of information available on cardiac gene regulation has stimulated the search for alternative approaches.

Introduction of exogenous DNA into germ cells and the creation of transgenic animals has greatly improved the study of gene regulation in a physiologically meaningful environment, as well as during development and organ differentiation,[6] but for practical purposes, this approach is limited to small mammals, the mouse in particular, because of the significant effort and costs related to the creation of transgene carriers and the fact that the vast majority of genetic knowledge in mammals originates from the mouse. Thus, the recent demonstration of gene transfer in vivo by simple injection of pure plasmid DNA into skeletal muscle of living mice[8] was a significant development that provided a convenient approach in the study of gene regulation and combined many of the advantages of the in vitro transfections and the transgenic models for short-term analyses. Varia-

This manuscript from Harvard Medical School was sent to Howard E. Morgan, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

From the Laboratory of Molecular and Cellular Cardiology, Howard Hughes Medical Institute and Department of Cardiology, Children's Hospital, Harvard Medical School (R. von H., R.J.S., V.M., B.N.-G.), Boston, and the Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, the New England Regional Primate Center (Y.-T.S., S.F.V.), Southborough, Mass.

Supported in part by National Institutes of Health grant HL-38070. R. von H. is a recipient of a fellowship of the Deutsche Forschungsgemeinschaft (Ha-1777/1-1). R.J.S. is an appointed trainee-fellow of the Bugher Foundation of the American Heart Association. B.N.-G. is an investigator of the Howard Hughes Medical Institute.

Address for correspondence: Bernardo Nadal-Ginard, MD, PhD, Department of Cardiology, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115.

Received October 13, 1992; accepted December 7, 1992.

tions of this principle for in vivo gene transfer have been used to deliver recombinant DNA[9–11] or in vitro genetically altered smooth muscle cells[12,13] to vascular endothelium via catheter, resulting in low levels of gene expression. In addition, this model of gene transfer in vivo offers a potential route for gene therapy in human disease.[14]

The potential to take up and express naked DNA introduced by simple injection is not limited to skeletal muscle. In the recent past, several laboratories have demonstrated the expression of naked recombinant DNA injected into the rat heart.[15–18] In this model, expression of the injected genes, as determined by histochemistry, appears to be localized around the injection channel,[15,16] with a small number of cells actually expressing the gene product.[17] The transfection efficiency appears to be higher with closed circular than with linear plasmid DNA.[15] Southern blot analysis of DNA obtained from skeletal muscle of mice injected with plasmid DNA provided the first evidence of the episomal localization of the gene construct.[8]

Rodents differ substantially in their cardiovascular physiology and pathophysiology from humans in the rate of metabolism, as reflected in the heart rate, in the pattern of cardiac contractile isoforms,[19,20] and in the induction of isoform switches during development and hypertrophy.[21,22] Moreover, although small mammals have been the model of choice for genetic and molecular analyses, large mammals, particularly the dog, have been the model of choice for physiological studies, especially when a large heart is necessary for the analysis of cardiac performance. These facts, together with the closer similarities between canine and human cardiac physiology, make this species the animal of choice for the analysis of gene regulation under experimental conditions that are physiologically meaningful and that could provide relevant insights into expression patterns prevailing in humans.

For all these reasons, we have developed a methodology to study gene expression in large mammals by injection of plasmid DNA into canine myocardium. Our data, which show that the predominant isoform of contractile proteins in the canine myocardium is, as in humans, the β-myosin heavy chain (β-MHC), further support the use of this model for studies relevant to human cardiovascular biology. Several important parameters relevant to the expression of injected gene constructs in this model have been characterized. The results demonstrate that the canine myocardium is an excellent receptor for injected genes and provides an ideal system for the analysis of gene expression in vivo under carefully monitored physiological conditions.

Material and Methods

Plasmids

MSV-CAT was created by fusing the coding sequence of the chloramphenicol acetyltransferase (CAT) gene[23] to the long terminal repeat of the mouse sarcoma virus (MSV). Rous sarcoma virus (RSV)–luciferase was described previously.[24] The series of deletions of the 5′ flanking region of the β-MHC included the −3,300rβ-MHC–CAT, −667rβ-MHC–CAT, −354rβ-MHC–CAT, and −215rβ-MHC constructs, which are genomic fragments of the rat β-MHC (rβ-MHC) gene from −3,300, −667, −354, −215, and −186 base pairs (bp) to +38 bp relative to the transcriptional start site cloned in front of the CAT gene.[25] Position −607 to +32 of the rat α-myosin heavy chain (rα-MHC) promoter sequence linked to the CAT gene is termed −607rα-MHC–CAT.[26] Nucleotide sequence −256 to +500 of the 5′ flanking sequence of the rat apolipoprotein A-I (ApoAI) fused to the CAT gene is termed −256ApoAI-CAT.[27]

Animal Preparation and Injection of Plasmid DNA

Fourteen adult mongrel dogs of either sex weighing between 20 and 26 kg were used for these experiments. Dogs were premedicated with xylazine (10 mg/kg i.m.), and general anesthesia was induced with thiamylal (10–20 mg/kg i.v.) and maintained with halothane (0.5–1.5% [vol]). Sterile technique was used, and the pericardium was exposed through a lateral thoracotomy at the fifth intercostal space. The pericardium was opened, and the heart was anchored with a suture through the apex. Up to thirty 4-mm^2 patches of Dacron were sewn to the epicardium to mark injection sites. After placement of the patches, circular plasmid DNA resuspended in 1× phosphate-buffered saline was injected through a 30-gauge needle inserted perpendicular to the epicardium. The incision was closed in layers, and the chest was evacuated. The animals were observed during recovery until fully conscious. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the "Guide for the Care and Use of Laboratory Animals" (Department of Health and Human Services, publication No. [NIH] 86-23).

Tissue Preparation

After 7 days, the animals were killed with an overdose of pentobarbital, and the heart was rapidly excised and placed in ice-cold saline. The labeled injection sites were excised as transmural blocks of myocardium, weighing 0.5–1.0 g, and immediately placed in liquid nitrogen. Tissue was stored at −80°C until further processing. Immediately before the CAT assay, tissue was homogenized in 1 ml homogenization buffer containing (mM) glycyl-glycine 25 (pH 7.8), $MgSO_4$ 15, EGTA 4 (pH 8.0), and dithiothreitol 1, as described previously.[18] The suspension was centrifuged at 6,000*g* for 15 minutes at 4°C, and the supernatant was used for further analysis. The supernatant was normalized for protein content as determined by the Bradford assay (Bio-Rad Laboratories, Richmond, Calif.) by the appropriate dilution with homogenization buffer.

CAT Assays

CAT assays were performed as previously described.[28] In brief, 10% of the supernatant normalized for protein content, 1 μl ^{14}C-labeled chloramphenicol (0.25 mCi), and 5 μl *n*-buturyl coenzyme A (5 mg/ml) were mixed and filled to a total volume of 125 μl with 250 mM Tris-HCl, pH 8.0. The reaction mixture was incubated at 37°C for 2 hours, which was in the linear range of the reaction. The acetylated chloramphenicol fraction of the suspension was extracted by adding 300 μl xylene. Suspensions were back-extracted twice with 250 mM Tris-HCl, pH 8.0. Aliquots of 200 μl were counted in scintillation fluid in a beta counter (model LS 6000IC, Beckman Instruments).

Luciferase Assay

Luciferase assays were performed as described elsewhere.[29] In summary, 10% of the supernatant normalized for protein content was brought to a volume of 100 μl with homogenization buffer (see "Tissue Preparation") and mixed with 360 μl reaction buffer containing (mM) glycyl-glycine 25 (pH 7.8), $MgSO_4$ 15, EGTA 4 (pH 8.0), dithiothreitol 1, KPO_4 15 (pH 7.8), and ATP 2, along with 0.3% Triton X-100. Light emission was measured in a monolight luminometer (1251 luminometer, LKE Wallac, Turqu, Finland) immediately after the addition of 0.2 mM D-luciferin to the reaction mixture. Light units are expressed as the integral of activity measured over 20 seconds. Only values within the linear range were included for analysis.

Data Analysis

All data are reported as mean±SEM. For statistical comparisons of CAT activity across time and regionally within the left ventricle, analysis of variance (ANOVA) was used. ANOVA was also used for promoter comparisons and comparison of injection techniques. When significant, intergroup comparisons were performed by unpaired *t* tests with the Bonferroni adjustment. Linear regression analysis was used to examine the correlation between CAT and luciferase activities in the cotransfection experiments. All analyses were performed on a Macintosh computer using STATVIEW II (Abacus Concepts Inc., Berkeley, Calif.) with $p<0.05$ considered significant.

Results

Expression of Gene Constructs Injected Into the Myocardium Follows Dose–Response Kinetics

To determine the efficiency and kinetics of expression of DNA injected into canine myocardium, we injected a constant volume of 200 μl containing increasing amounts of MSV-CAT plasmid DNA ranging from 10 to 300 μg per injection site into one dog heart. As depicted in Figure 1, an amount as little as 10 μg DNA resulted in a CAT signal nearly 10 times the background signal. In the range of 10–200 μg, the dose–CAT activity relation appeared linear ($y=0.2x+10.8$; $r^2=0.54$). Higher amounts of total DNA resulted in a plateau, reflecting a saturation kinetic of DNA uptake, transcription, or both. These results indicate that the canine myocardium has a large capacity for uptake of injected DNA over a very broad range of concentrations. However, the slope of the curve clearly indicates that the efficiency of expression is the highest at the lower concentrations. The reasons for this behavior are not known at this time. However, this finding stresses the requirement for internal standards when the efficiency of expression between different constructs and/or amounts of injected DNA are to be compared.

Amount of Injury Imposed on Injection Site Does Not Correlate With the Level of Gene Expression

Three different injection techniques were compared in one experiment to analyze the impact of injection-induced injury on the cardiac tissue and its relevance for the level of expression of the injected reporter gene constructs. Because it could be argued that the results presented here represent uptake by nonmyocyte cells

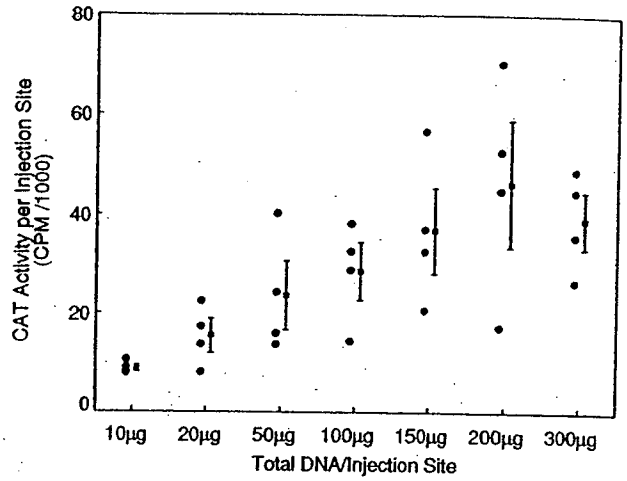


FIGURE 1. *Dose–response relation between amount of injected DNA and chloramphenicol acetyltransferase (CAT) activity. Scatterplot of CAT activity per injection site versus total amount of DNA (mouse sarcoma virus–CAT) per injection site is shown. Different concentrations of DNA were injected in a constant volume of 200 μl. The correlation equation for the concentrations between 10 and 200 μg is* $y=0.2x+10.8$; $r^2=0.54$. *Means (±SEM) are shown as solid squares (n=4 for each dose).*

and therefore are not meaningful for the study of cardiac muscle biology, we tested a reporter construct whose expression is restricted to muscle cells. Although, to date, there has been no published report identifying the pattern of myosin heavy chain expression in the canine myocardium, our RNA blot analysis indicates that, like in other large mammals, the normal adult isoform is β-MHC, with a low level of α-MHC (data not shown). For this reason, we chose to use previously characterized β-MHC promoter constructs for this analysis.[25] In the first group, 200 μl of −667rβ-MHC–CAT plasmid DNA solution were injected via one single injection. In the second group, 50 μl of the same concentration of DNA was injected four times per injection site. To account for differences in the distribution of the DNA solution in the tissue between those two groups, a third group was included in which 200 μl of the DNA solution containing the same amount of total DNA as the other two groups were injected via one injection, and three additional stabs with the needle (but without injection of DNA) were performed around the actual injection site. As depicted in Figure 2, there were no statistical differences in CAT activity between any of these groups (ANOVA, $p>0.05$). However, because of the apparent trend of higher expression in the group with 50 μl DNA injected four times and because of a lower standard deviation in this group, we used this injection technique for subsequent experiments.

Promiscuous and Tissue-Specific Reporter Gene Constructs Are Expressed Over Extended Periods of Time

To evaluate the stability and peak of the expression of injected recombinant gene constructs in canine myocardium, we killed animals at four different time points (days 3, 7, 14, and 21 after injection). Multiple injections of CAT gene constructs using either promiscuous

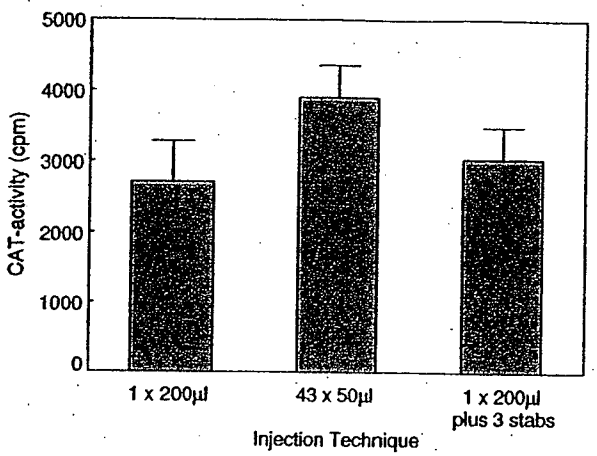


FIGURE 2. *Bar graph comparing three different injection techniques. rβ-MHC, rat β-myosin heavy chain; CAT, chloramphenicol acetyltransferase. A total amount of 50 µg of −667rβ-MHC–CAT was injected in each group. Values are mean±SEM (n=10 for each group).*

(MSV) or muscle-specific (−667rβ-MHC) promoters were performed (Figure 3). ANOVA for CAT activity within the MSV and −667rβ-MHC promoters was significant ($p<0.01$ for the MSV and −667rβ-MHC constructs). The overall temporal pattern of expression of the exogenous genes was similar between promiscuous and tissue-specific promoter constructs, with CAT activity already well detectable 3 days after injection, a peak at day 7, and a subsequent decline in CAT activity throughout day 21 ($p<0.01$ for MSV and $p<0.0001$ for −667rβ-MHC by unpaired t test). This pattern of expression at the protein level is likely to be an overestimate of duration of expression of the injected DNA because of the long half-life of the CAT protein, which is more than 50 hours in most cell types.[30] Therefore, the levels of expression shown in Figure 2 not only reflect the activity of the driving promoter but also reflect phenomena beyond the transcriptional level, e.g.,

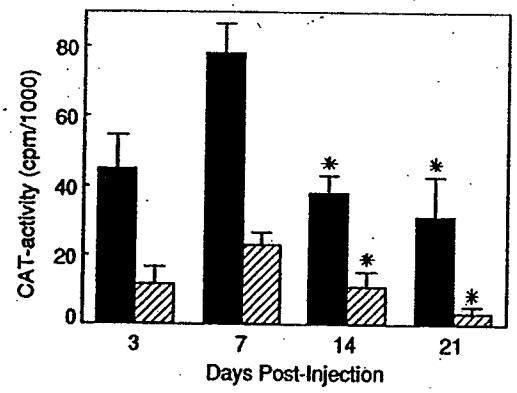


FIGURE 3. *Bar graph showing time course of expression of injected gene constructs. CAT, chloramphenicol acetyltransferase; rβ-MHC, rat β-myosin heavy chain. CAT activity versus days after injection for promiscuous (mouse sarcoma virus, solid bars) and muscle-specific (−667rβ-MHC, hatched bars) promoters driving the CAT reporter gene (compared with day 7, *p<0.01 for mouse sarcoma virus and *p<0.0001 for −667rβ-MHC by unpaired t test). Values are mean±SEM (n=5 for each time point).*

the half-life of the expressed protein as well as that of the injected episomal DNA.

Reporter Gene Constructs Are Evenly Expressed Throughout the Left Ventricular Wall

Since it was the purpose of our study to establish a practical model to compare the regulation of different recombinant gene constructs within the same animal by injecting at numerous sites into the canine myocardium, we compared the expression of a given construct at different locations throughout the myocardium. To account for regional differences in uptake and/or expression of foreign DNA by the cardiocytes, we injected the muscle-specific construct −667rβ-MHC–CAT in 24 different sites of the left ventricle as depicted in Figure 4. There were no detectable regional differences in CAT expression (ANOVA, $p>0.05$); however, because of the apparent trend toward decreased expression at the extreme base and apex of the left ventricle, we elected not to inject at those locations for subsequent experiments.

CAT Activity Is Approximately Threefold Higher in the Left Than in the Right Ventricle

One of the advantages of the canine versus the rodent model is the possibility to perform multiple injections also into the right ventricle. As shown in Figure 5, the expression of promiscuous as well as tissue-specific promoter constructs was found to be a third that observed in the left ventricle. This phenomenon could be based on the difference in wall thickness, with a smaller number of cells being transfected along the injection tract in the right ventricle. Alternatively, this may reflect a higher chance of leakage of injected DNA into the myocardial cavity during injections into the right ventricle.

The Heart Expresses Injected Reporter Gene Constructs One to Two Orders of Magnitude Higher Than Does the Skeletal Muscle

To compare the level of expression of injected gene constructs in the canine heart with other organs and possibly detect organ-related differences in the expression of exogenous genes, we performed injections of the promiscuous MSV-CAT and the muscle-specific β-MHC–CAT constructs into the quadriceps, a skeletal muscle of mixed fiber types. The values in Figure 5 are expressed as percentage of the expression of the according construct in the left ventricle. The expression of both the muscle-specific and promiscuous promoter constructs was, respectively, approximately one and two orders of magnitude lower in the skeletal muscle than in the left ventricle. As demonstrated before on mRNA levels in the rat, the β-MHC is most abundant in the soleus, a skeletal slow-twitch muscle, and also in the cardiac ventricle in hypothyroid animals.[31] Thus, the low level of expression of the β-MHC promoter construct may be due to the fact that we injected the DNA into a mixed-fiber muscle, in which the β-MHC protein is much less abundant than it is in slow-twitch fiber muscle. The reason for the lower expression of the promiscuous construct in the skeletal muscle compared with the heart is unknown.

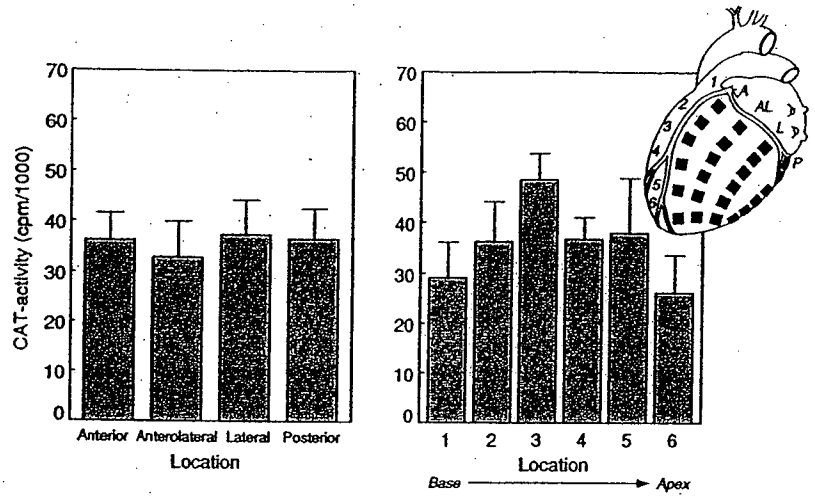


FIGURE 4. *Bar graphs showing regional expression pattern of injected gene constructs throughout the left ventricular wall. rβ-MHC, rat β-myosin heavy chain; CAT, chloramphenicol acetyltransferase; A, anterior; AL, anterolateral; L, lateral; P, posterior. Twenty-four injections of −667rβ-MHC–CAT were performed with four columns around the left ventricle, each comprising six injection sites ranging from base to apex (see drawing). Mean±SEM values of each column are shown in the left panel (n=6). Mean±SEM values of each row are shown in the right panel (n=4).*

Coinjection of a Control Gene Construct (RSV-Luciferase) Has Proven Useful in Monitoring the Transfection Efficiency in This Model

Cotransfection is used to control for transfection efficiency in vitro, and it has also been used in vivo,[18,32] but its usefulness in in vivo experiments has not been evaluated before. This is particularly important since, in contrast to cell culture studies, the transfected cell pool consists of a heterogeneous cell population, which may express the two gene constructs in a diverse pattern, rendering the coinjection useless. Two representative experiments were analyzed for correlation of CAT versus luciferase expression in this model, as depicted in Figure 6. In one experiment, the muscle-specific −667rβ-MHC–CAT construct was coinjected with the RSV-luciferase gene (Figure 6A). The long terminal repeat (LTR) of RSV (RSV-LTR) functions as a relative promiscuous promoter as determined previously, where transgenes directed by the RSV-LTR were highly expressed in tissue of mesodermal origin.[33,34] The correlation between CAT and luciferase activity was significant (r^2=0.8, slope=0.8±0.2, p<0.01). When two promiscuous promoter constructs (MSV-CAT and RSV-luciferase) were coinjected (Figure 6B), the linear regression analysis of CAT activity versus luciferase activity revealed an r^2 of 0.9, with a slope of 3.5±0.6 (p<0.005, n=6). This indicates that cotransfection is

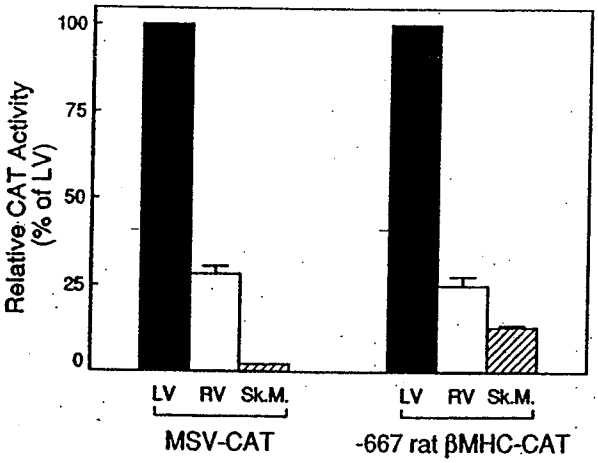


FIGURE 5. *Bar graph showing expression of promiscuous (mouse sarcoma virus [MSV]) or muscle-specific (−667 rat β-myosin heavy chain [−667 rat βMHC]) promoter constructs in the right ventricle (RV) and in skeletal muscle (Sk.M.). CAT, chloramphenicol acetyltransferase. Values (mean±SEM) are depicted as percent of expression of the same construct in the left ventricle (LV, 100%, solid bars). Open bar is RV (n=10 for MSV, n=8 for −667 rat βMHC). Hatched bar is skeletal muscle (n=10 for MSV, n=9 for −667 rat βMHC).*

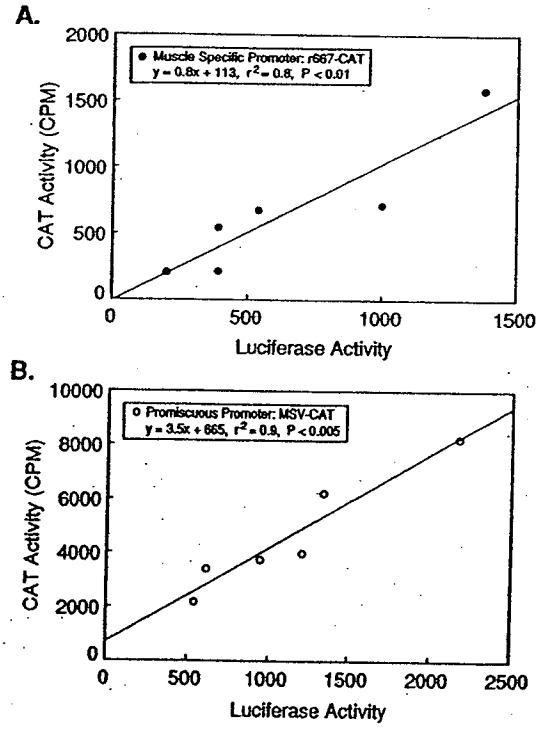


FIGURE 6. *Correlation of chloramphenicol acetyltransferase (CAT) to luciferase activity in coinjection experiments. r667-CAT, −667 rat β-myosin heavy chain–CAT; MSV-CAT, mouse sarcoma virus–CAT. Scatterplots of CAT activity (counts per minute) versus luciferase activity (light units) are shown. One hundred micrograms of a tissue-specific (r667-CAT, closed circles, top panel) or a promiscuous (MSV-CAT, open circles, bottom panel) reporter gene construct was coinjected with 20 μg control gene construct (Rous sarcoma virus–luciferase). The regression functions are as indicated.*

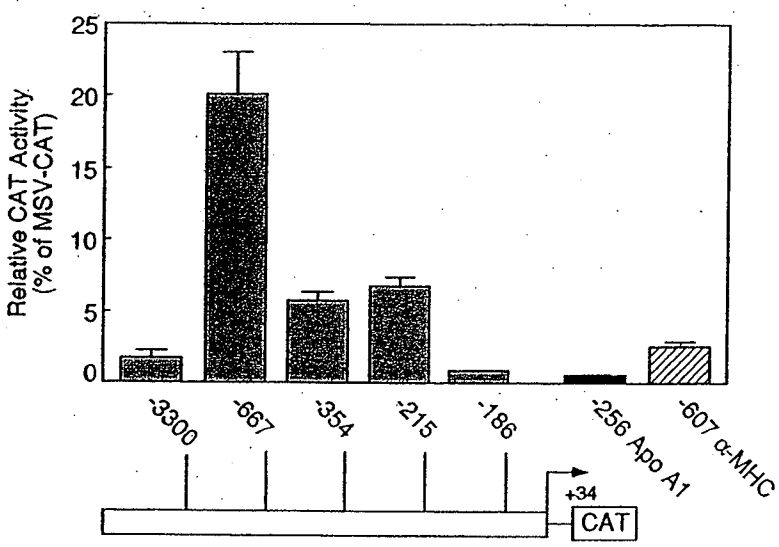


FIGURE 7. *Bar graph mapping the 5′ flanking region of the β-myosin heavy chain (β-MHC) gene in vivo. CAT, chloramphenicol acetyltransferase; MSV, mouse sarcoma virus; Apo A1, apolipoprotein A-I; α-MHC, α-myosin heavy chain. A series of deletions of the upstream region of the rat β-MHC gene ranging from −3,300 to −186 relative to the transcription start site were cloned in front of the CAT gene and injected into the canine myocardium. For comparison −607 rat α-MHC–CAT and −256 Apo A1–CAT were also injected. Reporter gene construct (100 μg) was coinjected with 20 μg control gene construct (Rous sarcoma virus–luciferase). CAT activity was corrected for luciferase activity and is expressed in percentage of MSV-CAT. Open bars are β-MHC–CAT constructs (n=6–10). Hatched bar is the α-MHC–CAT construct (n=10). Solid bar is the Apo A1–CAT construct (n=10). See text for statistical analysis.*

meaningful and necessary to account for transfection efficiency in vivo; therefore, differences in expression of gene constructs may be attributed to differences in the regulation of expression of these constructs as long as values are normalized for the activity of the cotransfected gene. This analysis also reveals the feasibility of coinjecting a tissue-specific promoter construct with a promiscuous promoter construct to control for transfection efficiency.

Injection of Reporter Gene Constructs Into Canine Myocardium as a Useful Method to Detect Regulatory Gene Sequences In Vivo

After characterization of many important parameters relevant for the regulated expression of injected reporter gene constructs, we addressed the question of the feasibility of this model for mapping promoter sequences and thus identifying regulatory gene sequences in vivo. Constructs using serial deletions of the 5′ flanking region of the β-MHC gene cloned in front of the CAT reporter gene were used (Figure 7). As a negative control, we used a construct containing the nucleotide sequence −256 to +397 relative to the transcription start site of the ApoAI gene, which has been shown to be expressed in hepatocytes specifically.[27] Activity of the different β-MHC constructs was compared by ANOVA ($p=0.001$). All six possible pairwise comparisons were made and found to be significant ($p<0.005$), except −354rβ-MHC versus −215rβ-MHC. The most active construct was the −667rβ-MHC–CAT reporter gene construct. The marked difference in activity compared with the other β-MHC–CAT constructs agrees with the presence of a positive regulatory element between positions −667 and −354 relative to the transcription start site. Further deletion of the β-MHC gene promoter to position −186 relative to the transcription start site (−186rβ-MHC–CAT) resulted in a sharp decline of the CAT activity to a level that was barely above that of the negative control construct (−256ApoAI-CAT), indicating another positive regulatory element important for basal transcription between positions −215 and −186 relative to the transcription start site. A repressor element may be located further upstream, as implied by the drop of activity of the −3,300rβ-MHC–CAT construct to approximately one fourth of the activity of the −354 and −215rβ-MHC–CAT constructs. The −607rα-MHC–CAT construct, which is the most active tissue-specific construct in rat cardiocytes as demonstrated by transfection assays of primary cell cultures,[35] was approximately sixfold less active than the −667rβ-MHC–CAT. This result was expected, given the relative level of endogenous β- and α-MHC mRNA expressed in large mammals versus small mammals.

Taken together, our observations demonstrate that direct DNA injection into the canine myocardium is a practical and efficient method to study gene regulation in the intact animal.

Discussion

Our study indicates the feasibility of investigating the regulated expression of injected gene constructs in vivo in the heart of large mammals, specifically the canine myocardium. We demonstrate that pure plasmid DNA can be injected into the myocardial wall of dogs without any side effects. Electrographic monitoring and intraarterial blood pressure measurements performed postoperatively over several subsequent days in a number of dogs revealed only transient tachyarrhythmias in the first 1–2 hours after surgery, but otherwise no cardiac malfunction due to the injection procedure was detected. The efficiency of expression of gene constructs injected into canine myocardium compares favorably with other transfection methods.

In the first series of experiments, we defined parameters important for direct injection of reporter gene constructs into the canine myocardium. We show that expression of injected gene constructs is dose dependent and has features of a saturation kinetic at doses above 200 μg per injection site. To some extent, this has been demonstrated before in the skeletal muscle of mice by injecting three different doses of plasmid DNA ranging from 10 to 100 μg,[8] although no saturation kinetics were demonstrated.

The analysis of the time course of the expression of gene constructs in our model revealed a pattern similar to other investigations,[8,15] although these other studies were performed in rodents. The reason for the decline in the level of gene expression after 7 days is not known, but Acsadi et al[17] have suggested that injected plasmid DNA remains episomal and that the DNA itself is lost with time because of rapid degradation. One also might speculate that cells harboring the CAT gene product might be eliminated by immunological processes. This hypothesis is favored by recent experiments conducted on nude (immunosuppressed) rats, which showed a prolonged expression of an injected luciferase construct compared with normal rats.[17] Data obtained from transgenic animals also indicate that the expression of the transgene may induce immunological responses.[36] However, other reports were able to demonstrate a rather long-lasting gene expression of 19 months in rodents after in vivo gene transfer into skeletal muscle[37] and of 60 days after injection into rat hearts.[15]

The local pattern of exogenous gene expression has not been addressed before because of the small size of the species investigated. In the present study, we find no regional differences in expression for a muscle-specific promoter construct throughout the left ventricular wall. This renders the canine model suitable for comparison of different injected gene constructs within one animal, thus reducing the interindividual variability, whereas a large pool of animals is required in studies undertaken on rodents to gain statistical significance.[38] In contrast, we observed a reduction of expression in the right ventricle to approximately one third of the left ventricle, which is probably related to the differences in wall thickness and thus the number of cells, which can be transfected along the needle tract.

The histological distribution of cell transfection has been addressed in several reports either by analysis of expression in different areas around the injection site[15] or by use of constructs containing the coding sequence of the *Escherichia coli* lacZ gene.[16,17] Apparently only cells in the direct vicinity of the needle tract are transfected. This observation raised the question about the mechanism of DNA uptake in this model, since the localization around the needle tract favors the hypothesis of DNA uptake through leaking cell membranes induced by injury. However, our data reported here do not favor this hypothesis. The comparison of three different injection techniques with different degrees of injury imposed on the cardiac tissue did not reveal a positive correlation between the degree of injury and DNA uptake or expression. Since the expression pattern of injected reporter gene constructs seems to display tissue-specific gene regulation mechanisms (Figure 7), it is unlikely that macrophages, chemotactically attracted to the injection site and supposedly able to incorporate DNA molecules by phagocytosis, contribute significantly to the expression of the injected reporter gene constructs. However, the exact mechanism of uptake of injected DNA remains elusive. It has been reported elsewhere that administration of DNA through multiple injections decreased the expression or uptake of the injected gene constructs.[38] Since this observation is not in agreement with our results, further studies have to be undertaken to elucidate the mechanism of uptake of exogenous DNA constructs into mammalian cells in vivo.

Both, promiscuous and muscle-specific promoter constructs demonstrated a much lower level of expression in the skeletal muscle than in the heart. This observation is consistent with another report in which the promiscuous RSV-CAT, used for coinjection, was expressed approximately 20-fold less in skeletal muscle than in the heart of rats.[18] The reason for this difference in expression is not known and is the subject of speculation. The marked difference in expression of the promiscuous MSV-CAT promoter construct between cardiac and skeletal muscle is surprising if one bears in mind the generally known high level of expression of this construct in transfection assays of all cell types studied so far. In contrast to cells in culture, which do not underlie physiological control mechanisms, the expression of injected plasmid DNA in our model might reflect differences between certain organs, regarding the prevailing physiological regulation pattern. The rhythmical contraction of the heart with the concomitant alteration of the myocardial wall stress or other regional differences in the neurohormonal regulation of organ function might account for the observed difference of expression of both constructs in vivo. The differences in the structure of the tubule system between cardiac and skeletal muscle cells might be related to a different efficiency of DNA uptake between these two cell types. As for the β-MHC reporter construct, it also may reflect the difference in abundance of the β-MHC protein between cardiac and mixed fiber muscle as has been shown before.[31] However, more work will be required to uncover the basis for this phenomenon.

Although coinjection of a second gene construct to account for transfection efficiency, thereby reducing the variability inherent in transfection assays of cell cultures, is very common, its usefulness in in vivo gene transfer experiments has not been analyzed before. Our results indicate a high degree of correlation between expression of both injected genes, independent of the tissue specificity of the driving promoter. Thus, cotransfection in this model has proven to be useful.

To assess the usefulness of direct injection of DNA into canine myocardium to identify regulatory gene sequences important for in vivo expression (promoter mapping), we injected constructs containing a series of deletions of the 5′ flanking region of the β-MHC gene. Our data are in general agreement with results obtained from transfection assays of skeletal and cardiac muscle cells.[25] Notably, the $-667r\beta$-MHC–CAT construct seems to be more active in the heart than in skeletal muscle cells, as one can see by comparing the relative activity of the -667 with the -354 and the $-215r\beta$-MHC–CAT construct in the heart and in transfected skeletal muscle cells.[25] The reason for this observation is unknown, but it might reflect the existence of a positive regulatory element between nucleotide positions -667 and -354, which is recognized specifically in cardiocytes and acts cooperatively with other positive regulatory elements further downstream from the β-MHC gene promoter, one of which may be located between positions -215 and -186 relative to the transcription start site, as indicated by the marked drop of CAT activity induced by deletion of this sequence. This is in agreement with studies performed on cell cultures,

in which the same sequence has been found to be important for the basal activity of the β-MHC promoter in Sol8 myotubes.[25] In general, these findings prove the usefulness of this method in the identification of regulatory gene sequences in vivo and possibly their importance in the induction of pathophysiological conditions.

In summary, injection of recombinant gene constructs into canine myocardium appears to be a practical and efficient model for studying the regulated gene expression in the heart of large mammals. It allows for better extrapolation to humans than methods using small mammals, as have been done so far for this type of analyses. Furthermore, this model holds promise to serve as a tool to manipulate the cardiac phenotype.

Acknowledgments

The help of Reid Thompson in providing us with numerous β-myosin heavy chain constructs is gratefully acknowledged. We thank Cheryl Miller, DVM, for her help in taking care of the animals.

References

1. LaPointe MC, Wu J, Greenberg B, Gardner DG: Upstream sequences confer atrial-specific expression of the human atrial natriuretic factor gene. *J Biol Chem* 1988;263:9075–9078
2. Wu J, LaPointe MC, West BL, Gardner DG: Tissue-specific determinants of human atrial natriuretic factor gene expression in cardiac tissue. *J Biol Chem* 1989;264:6472–6479
3. Henderson SA, Spencer M, Sen A, Kumar C, Siddiqui MAQ, Chien KR: Structure, organization and expression of the rat cardiac myosin light chain-2 gene. *J Biol Chem* 1989;264:18142–18148
4. Rosenzweig A, Halazonetis TD, Seidman JG, Seidman CE: Proximal regulatory domains of rat atrial natriuretic factor gene. *Circulation* 1991;84:1256–1265
5. Kariya K, Karns LR, Simpson PC: Expression of a constitutively activated mutant of the β-isozyme of protein kinase C in cardiac myocytes stimulates the promoter of the β-myosin heavy chain isogene. *J Biol Chem* 1991;266:10023–10026
6. Field LJ: Atrial natriuretic factor-SV40 T antigen transgenes produce tumors and cardiac arrhythmias in mice. *Science* 1988;239:1029–1032
7. Rockman HA, Ross SR, Harris AN, Knowlton KU, Steinhelfer ME, Field LJ, Ross J, Chien KR: Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. *Proc Natl Acad Sci U S A* 1991;88:8277–8281
8. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL: Direct gene transfer into mouse muscle in vivo. *Science* 1990;247:1465–1468
9. Lim CS, Chapman GD, Gammon RS, Muhlestein JB, Bauman RP, Stack RS, Swain JL: Direct in vivo gene transfer into the coronary and peripheral vasculatures of the intact dog. *Circulation* 1991;83:2007–2011
10. Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ: Recombinant gene expression in vivo within endothelial cells of the arterial wall. *Science* 1989;244:1342–1344
11. Flugelman MY, Jaklitsch MT, Newman KD, Casscells W, Bratthauer GL, Dichek DA: Low level in vivo gene transfer into the arterial wall through a perforated balloon catheter. *Circulation* 1992;85:1110–1117
12. Plautz G, Nabel EG, Nabel GJ: Introduction of vascular smooth muscle cells expressing recombinant genes in vivo. *Circulation* 1991;83:578–583
13. Wilson JM, Birinyi LK, Salomon RN, Libby P, Callow AD, Mulligan RC: Implantation of vascular grafts lined with genetically modified endothelial cells. *Science* 1989;244:1344–1346
14. O'Brien TX, Hunter JJ, Dyson E, Chien KR: Heart-to-heart: New approaches for gene transfer in the myocardium. *Circulation* 1991;86:2133–2136
15. Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA: Behavior of genes directly injected into the rat heart in vivo. *Circ Res* 1991;70:193–198
16. Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM: Expression of recombinant genes in myocardium in vivo after direct injection of DNA. *Circulation* 1990;82:2217–2221
17. Acsadi G, Jiao S, Agnes J, Duke D, Williams P, Chong W, Wolff JA: Direct gene transfer and expression into rat heart in vivo. *New Biol* 1991;3:71–81
18. Kitsis RN, Buttrick PM, NcNally EM, Kaplan ML, Leinwand LA: Hormonal modulation of a gene injected into rat heart in vivo. *Proc Natl Acad Sci U S A* 1991;88:4138–4142
19. Lompre AM, Mercardier C, Wisnewsky P, Bouveret D, Pantaloni D, D'Albis A, Schwartz K: Species and age dependent changes in relative amounts of cardiac myosin isoenzymes in mammals. *Dev Biol* 1981;84:286–291
20. Lompre AM, Nadal-Ginard B, Mahdavi V: Expression of the cardiac ventricular α- and β-myosin heavy chain genes is developmentally and hormonally regulated. *J Biol Chem* 1984;259:6437–6446
21. Lompre AM, Schwartz K, D'Albis A, Lacombe G, Thiem NV, Swynghedauw B: Myosin isoenzyme redistribution in chronic heart overload. *Nature* 1979;282:105–107
22. Izumo S, Lompre AM, Matsuoka R, Koren G, Schwartz K, Nadal-Ginard B, Mahdavi V: Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. *J Clin Invest* 1987;79:970–977
23. Gorman CM, Moffat LF, Howard BH: Recombinant genomes which express cloramphenicol acetyl transferase in mammalian cells. *Mol Cell Biol* 1982;2:1044–1051
24. De Wet JR, Wood KV, DeLuca M, Helinski DR, Subramani S: Firefly luciferase gene: Structure and expression in mammalian cells. *Mol Cell Biol* 1987;7:725–737
25. Thompson WR, Mahdavi V, Nadal-Ginard B: A myoD1-independent muscle-specific enhancer controls the expression of the beta-myosin heavy chain gene in skeletal and cardiac muscle cells. *J Biol Chem* 1992;266:22678–22688
26. Mahdavi V, Chambers AP, Nadal-Ginard B: α- and β-myosin heavy chain genes are organized in tandem. *Proc Natl Acad Sci U S A* 1984;81:2626–2630
27. Widom RL, Ladias JAA, Kouidou S, Karathanasis SK: Synergistic interactions between transcription factors control expression of the apolipoprotein AI gene in liver cells. *Mol Cell Biol* 1991;11:677–687
28. Seed B, Sheen J-Y: A simple phase-extraction assay for chloramphenicol acetyl transferase activity. *Gene* 1988;67:271–277
29. Brasier AR, Tate JE, Habener JF: Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques* 1989;7:1116–1122
30. Thompson JF, Hayes LS, Lloyd DB: Modulation of firefly luciferase, stability and impact on studies of gene regulation. *Gene* 1991;103:171–177
31. Izumo S, Nadal-Ginard B, Mahdavi V: All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science* 1986;231:597–600
32. Parmacek MS, Vora AJ, Shen T, Barr E, Jung F, Leiden JM: Identification and characterization of a cardiac-specific transcriptional regulatory element in the slow/cardiac troponin C gene. *Mol Cell Biol* 1992;12:1967–1976
33. Overbeck PA, Lai SP, Van Quill KR, Westphal H: Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. *Science* 1986;231:1574–1577
34. Swain JL, Stewart TA, Leder P: Parental legacy determines methylation and expression of an autosomal transgene: A molecular mechanism for parental imprinting. *Cell* 1987;50:719–727
35. Mahdavi V, Koren G, Michaud S, Pinset C, Izumo S: Identification of the sequences responsible for the tissue-specific and hormonal regulation of the cardiac myosin heavy chain genes, in *Cellular and Molecular Biology of Muscle Development.* New York, Alan R Liss, Inc, 1989, pp 369–379
36. Tang D, DeVit M, Johnston SA: Genetic immunization is a simple method rot eliciting an immune response. *Nature* 1992;356:152–154
37. Wolff JA, Chong W, Acsadi G, Williams P: Expression of genes injected into mouse muscle in vivo. *Hum Mol Genet* 1992;1:53–63
38. Wolff JA, Williams P, Acsadi G, Jiao S, Jani A, Chong W: Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques* 1991;11:474–485

EXHIBIT O

Direct In Vivo Gene Transfer Into Porcine Myocardium Using Replication-Deficient Adenoviral Vectors

Brent A. French, PhD; Wojciech Mazur, MD; Robert S. Geske, BA; Roberto Bolli, MD

Background Efficient methods of introducing genes into myocardial cells must be developed before local somatic cell gene therapy can be implemented against myocardial disease. Although adenoviral (Ad5) vectors have been used to target rodent hearts and plasmid DNA has been directly injected into the myocardium of rats and dogs, the amounts of recombinant protein produced by these procedures have not been reported, and adenoviral vectors have not been used in large mammalian hearts.

Methods and Results Replication-deficient recombinant adenoviral vectors carrying either the luciferase or *lacZ* reporter genes were injected directly into the ventricular myocardium of adult domestic swine for evaluation of reporter gene expression. This procedure did not affect regional myocardial function as assessed by systolic wall thickening using ultrasonic crystals. Luciferase activity was detected 3 days after injection, increased markedly at 7 days, and then declined progressively at 14 and 21 days. Luciferase production was comparable in the right and left ventricular walls and increased with increasing amounts of virus, reaching 61±21 ng at the highest dose examined (3.6×10^9 plaque-forming units). The injection of 200 μg of plasmid DNA (pRSVL) produced levels of luciferase comparable to 1.8×10^8 plaque-forming units of recombinant Ad5; however, when normalized to the number of genes injected, the adenovirus was 140 000 times more efficient than plasmid DNA. Histochemical analysis of β-galactosidase activity produced by a second Ad5 vector demonstrated that nearly all (>95%) of the stained cells were cardiomyocytes and that the percentage of cardiomyocytes infected by the virus could be quite high in microscopic regions adjacent to the needle track (up to 75% in fields of 60 to 70 cells); however, Ad5-infected cells were rarely observed farther than 5 mm from the injection site. Furthermore, the Ad5 vector induced pronounced leukocytic infiltration that was far in excess of that seen after injection of vehicle alone.

Conclusions This study demonstrates for the first time that direct intramyocardial injection of replication-deficient adenovirus can program recombinant gene expression in the cardiomyocytes of a large animal species with relevance to human physiology. The efficiency of adenovirus-mediated gene transfer is far superior to that of plasmid DNA injection, and this method appears to be capable of producing more recombinant protein. However, the cell-mediated immune response to the Ad5 vector and the limited distribution of reporter gene expression suggest that less immunogenic recombinant vectors and more homogeneous administration methods will be required before Ad5 vectors can be successfully used for phenotypic modulation. (*Circulation*. 1994;90:2414-2424.)

Key Words • genetics • myocardium • adenoviruses • leukocytes

Gene therapy has recently emerged as a novel approach that may have important applications in the treatment of human disease.[1,2] In the field of cardiology, much interest has focused on developing techniques to introduce recombinant genes directly into the vasculature and the heart.[3] Although several studies have demonstrated successful gene transfer into the ventricular myocardium of rats using direct injection of plasmid DNA,[4-7] the number of myocytes transfected with this method appears to be too small for successful gene therapy application.[7] A study by von Harsdorf et al[8] demonstrated that intramyocardial injection of plasmid DNA can be applied to the intact dog, that it is suitable for studying the transcriptional regulation of cardiac-specific promoters, and that multiple DNA injections can be made in the same canine heart, thus increasing the yield of data from each animal. These reports[4-8] provided relative measurements of the levels of recombinant gene product obtained following direct intramyocardial injection, and many of them assessed the distribution of reporter gene expression using a histochemical assay for β-galactosidase activity.[4,5] However, these studies did not report the absolute amounts of reporter protein produced following the direct injection of plasmid DNA into intact myocardium.

Recombinant vectors based on adenovirus serotype 5 (Ad5) represent an alternative means of introducing genes into the cardiovascular system.[3] Reporter gene expression following direct introduction of plasmid DNA has been demonstrated in skeletal muscle,[9] cardiac muscle,[4-8] and even the vascular wall[10]; however, this method fails to produce significant levels of recombinant protein in most other tissues.[5] In contrast, Ad5-mediated gene transfer has been demonstrated in a wide variety of tissues following direct in vivo administration.[11-16] Furthermore, although Acsadi et al[5] reported that plasmid DNA directs the expression of recombinant protein for a limited time (<25 days) in a limited number of cardiomyocytes (~100), replication-

Received April 8, 1994; accepted June 10, 1994.

From the Molecular Cardiology Unit (B.A.F., W.M.) and Experimental Animal Laboratory (R.B.), Section of Cardiology, Department of Medicine, and the Center for Comparative Medicine (R.S.G.), Baylor College of Medicine, Houston, Tex.

The guest editor was J. David Bristow, MD.

Correspondence to Brent A. French, PhD, Section of Cardiology, Department of Medicine, Baylor College of Medicine, One Baylor Plaza, Rm 543-E, Houston, TX 77030.

deficient Ad5 vectors have been reported to mediate widespread and long-term gene transfer.[11]

The molecular mechanisms by which Ad5 vectors accomplish endocytosis, endosomal disruption, and nuclear entry[17] make them very efficient at accomplishing gene transfer and thus attractive vectors for delivering somatic cell gene therapy to intervene in human disease states. Protocols using replication-deficient Ad5 vectors have received approval from the Recombinant DNA Advisory board for limited investigational use in patients with cystic fibrosis,[12] and Ad5 vectors that direct the expression of the low-density lipoprotein (LDL) receptor have been shown to temporarily accelerate cholesterol clearance in healthy mice.[18] The potential advantages of Ad5 vectors versus plasmid DNA make the former approach attractive[3]; however, a number of important parameters must be characterized before recombinant Ad5 can be considered a reliable vector for gene therapy, including the magnitude and duration of recombinant gene expression, the identity of the targeted host cells, the distribution of transfected cells, and the response of the host to Ad5-mediated gene transfer.

Stratford-Perricaudet et al[11] first demonstrated direct gene transfer into intact myocardium by Ad5 vectors following the intravenous injection of mice. They reported that Ad5-mediated gene transfer into the hearts of adult mice was relatively inefficient when compared with neonatal mice, but a quantitative comparison was not provided. This may in part be due to the fact that most studies using Ad5 vectors to deliver genes to the intact myocardium[11,16,19] have used a histochemical assay for β-galactosidase,[20] which identifies infected cells by a distinctive blue stain. The histochemical assay provides an efficient means of identifying cells that express the *lacZ* reporter gene; however, it does not provide a quantitative assessment of recombinant gene expression, nor does it provide an accurate estimate of the percentage of cells transfected in the entire organ (unless it includes an exhaustive quantitative analysis of serial sections). A number of spectrophotometric and chemiluminescent assays have been used to measure β-galactosidase activity in tissue extracts; however, these assays cannot distinguish between the recombinant β-galactosidase and the endogenous β-galactosidase activity, which is elevated in response to injury.[21] In this regard, a recent study of β-galactosidase activity following direct injection of Ad5 vectors into the rat heart has reported discrepancies between the histochemical and quantitative results, and it was concluded that the quantitative assay for β-galactosidase is relatively insensitive for assessing gene transfer.[16]

In summary, previous studies have demonstrated that direct in vivo gene transfer into ventricular cardiomyocytes is possible using plasmid DNA or Ad5 vectors; however, these studies have not reliably measured the amount of recombinant protein produced, so it has not been possible to make accurate, quantitative comparisons between the efficiencies of plasmid- and Ad5-mediated gene transfer. Such quantitative information is of critical importance in assessing the potential of these systems to deliver gene therapy. Furthermore, the previous studies of Ad5-mediated gene transfer to the myocardium were performed in species such as mice,[11] rabbits,[19] or rats,[16] in which cardiovascular physiology differs from human physiology in many important respects and accurate assessment of in vivo cardiac function is problematic. If manipulations of cardiac phenotype via Ad5 vectors are to be correlated with sophisticated physiological measurements, a larger experimental animal must be used. We chose the porcine model[22] for this purpose because the coronary anatomy of swine is similar to that of humans, the vasomotor responses of swine are similar to those of humans, atherosclerosis can be induced in swine as in humans, and the size of the porcine heart facilitates experimental manipulations and enables sophisticated measurements of regional function and flow.

To provide accurate quantitative data on the levels of recombinant gene expression resulting from the direct injection of recombinant Ad5 vectors into intact myocardium, we constructed a replication-deficient adenovirus carrying the luciferase reporter gene. The advantages of the luciferase reporter system include the extreme sensitivity of the luminometric assay[23] and the total absence of background activity in any mammalian tissue. Using this recombinant adenovirus, we assessed the potential of Ad5 vectors to mediate gene transfer after direct injection into the right and left ventricles of living swine. In addition, we determined the duration of Ad5-mediated gene expression and the efficiency of Ad5 vectors relative to the plasmid-based approach of direct DNA injection. A second Ad5 vector directing the expression of *Escherichia coli* β-galactosidase was used to characterize the identity and distribution of Ad5-infected cells after direct intramyocardial injection. Finally, immunohistochemical studies were performed to characterize the inflammatory response observed after intramyocardial injection of Ad5 vectors.

Methods

Recombinant Vectors

The replication-deficient adenovirus in which the Rous sarcoma virus long terminal repeat promotes the transcription of the luciferase reporter gene (Ad5/RSV/GL2) was generated by the method of McGrory et al[24] from the homologous recombination of two plasmid components (pJM17 and pXCJL.1/RSV/GL2) following the cotransfection of the 293 host cell line.[25] The resulting virus was replication deficient due to the deletion of critical E1 genes. The recombinant Ad5 vector can replicate in the 293 cell line because this permissive host contains an integrated copy of the viral E1 genes. The β-galactosidase reporter virus (Ad5/HCMV/*LacZ*) was generously provided by F.L. Graham and A. Bett (McMaster University, Ontario, Canada). In this replication-deficient Ad5 derivative, the human cytomegalovirus IE promoter transcribes the *E. coli lacZ* reporter gene.[26] The Ad5/RSV/GL2 and Ad5/HCMV/*LacZ* viruses were plaque-purified and propagated in 293 cells according to published protocols.[25] A cell lysate preparation of Ad5/RSV/GL2 with a plaque assay titer of 2×10^9 was used to perform the experiments summarized in Figs 1 and 2 and as indicated in Fig 3, while doubly cesium chloride–banded preparations of Ad5/RSV/GL2 and Ad5/HCMV/*LacZ* with plaque assay titers of 1 to 4×10^{10} were used for the balance of the experiments. The plasmid pRSVL, kindly provided by S. Subramani,[27] carries the long terminal repeat of the Rous sarcoma virus, the firefly luciferase cDNA, the SV40 small-t intron, and the SV40 polyadenylation signal.

Animal Preparation and Injection of Virus

This study was performed in accordance with the guidelines of the Animal Protocol Review Committee of the Baylor College of Medicine and with the "Guide for the Care and Use

of Laboratory Animals" (Department of Health and Human Services, publication no. [NIH] 86-23). Male domestic swine weighing 28.5±1.6 kg were premedicated with acepromazine maleate (1 mg/kg IM) and atropine (0.02 mg/kg IM). Seven pigs were used for injection of gene transfer reagent and three pigs served as controls. Anesthesia was induced with methohexital sodium (4 to 8 mg/kg) and maintained via ventilation with 0.5% to 1.0% methoxyflurane. With sterile technique, a thoracotomy was performed in the left fifth intercostal space, the pericardium was opened, and the heart was lifted slightly with gauze to facilitate injection. A 4×4-cm rectangular grid was mapped onto the left ventricle, and injections were performed near intersecting lines to avoid epicardial vessels while maintaining a 1-cm distance between injection sites. All intramyocardial injections were 100 μL in volume and were performed with 1-mL syringes and 27-gauge needles. Injections into the left ventricular wall were made perpendicular to the surface of the heart with needles that were fitted with insertion guards to ensure a consistent injection depth of 4 mm. The thinner right ventricular wall was injected at an oblique angle and at a depth of 1 to 2 mm to prevent the needle from penetrating into the ventricular cavity. The location of each injection site was marked with a suture onto which a miniature plastic placard was secured. In three of the experimental pigs, two Doppler ultrasonic crystals[28] were sutured to the epicardial surface to identify specific Ad5/RSV/GL2 injection sites and to assess regional myocardial function as systolic thickening fraction. Two Doppler probes were sutured onto corresponding regions of the myocardium in each of three control swine that did not receive intramyocardial injections.

The histochemical studies involving the Ad5 vector carrying the *lacZ* reporter gene (Ad5/HCMV/*LacZ*) were designed to evaluate the distribution of gene transfer that resulted from injecting high doses of virus in close proximity to one another. Accordingly, a 5-cm-long monofilament template was secured onto the epicardium of the anterolateral wall of the left ventricle parallel to the atrioventricular groove using sutures to demarcate the middle and two ends. Ten injections, each containing 1.4×10^9 plaque-forming units (pfu) in a 100-μL volume, were made at 2.5-mm intervals along the anterior half of this linear template, while a second set of 10 injections were made at 2.5-mm intervals along the lateral half using vehicle alone (100-μL volumes of 1× phosphate-buffered saline [PBS], 1% sucrose). After the direct injection of gene transfer reagent and vehicle control, a small plastic tube was placed in the thorax to evacuate air and fluid after surgery, and a Tygon catheter was introduced into the femoral artery to record arterial pressure. The incision was closed in layers, and each animal was observed carefully during recovery. Three to 21 days after surgery, the animals were anesthetized with pentobarbital sodium (35 mg/kg IV) and killed with a bolus of KCl for analysis of reporter gene activity.

Luciferase Assay

The heart of each animal was removed and placed in ice-cold PBS. The left ventricular cavity was opened, and the left ventricular wall was separated from the right ventricle, the atria, and the large vessels. Each injection site was identified by its sutured placard (or ultrasonic Doppler probe). A brass cork-borer 1 cm in diameter (Fisher Scientific Co) was then used to remove a transmural cylindrical core of myocardium whose axis was defined by the needle injection path and whose center corresponded to the labeled injection site. Pilot studies had established that nearly all of the luciferase activity was located within a radius of 3 mm from the needle track, and essentially no activity could be detected >5 mm from the needle track. Each sample was temporarily stored in ice-cold PBS until mincing for homogenization in lysis buffer (25 mmol/L Tris-phosphate, pH 7.8, 2 mmol/L dithiothreitol, 2 mmol/L EDTA, 10% glycerol, and 1% Triton X-100). Homogenates were centrifuged at 3000*g* for 10 minutes, and the resulting supernatants were further clarified by means of a second centrifugation at 12 000*g* for 5 minutes. Duplicate enzymatic assays for luciferase activity[23] were performed with 20-μL samples of cleared supernatant using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). A 100-μL aliquot of assay reagent [20 mmol/L Tricine, pH 7.8, 1.07 mmol/L $(MgCO_3)_4Mg(OH)_2\cdot 5H_2O$, 2.67 mmol/L $MgSO_4$, 0.1 mmol/L EDTA, 33.3 mmol/L dithiothreitol, 270 μmol/L coenzyme A, 470 μmol/L D-luciferin, and 530 μmol/L ATP] was added to each 20-μL sample, and light production over a period of 30 seconds was measured using the microprocessor-controlled photon counter. Background values from samples injected with the PBS vehicle (equivalent to background from lysis reagent) were subtracted and relative light units were converted to mass units (pg) using calibration curves generated from parallel reactions performed with certified luciferase control standard (Analytical Luminescence Laboratory). Values are reported as the total mass of active luciferase recovered from each injection site.

Histochemical Assay for β-Galactosidase

The histochemical assays for β-galactosidase were performed 7 days after the direct injection of Ad5/HCMV/*LacZ* into the left ventricle. Gross histological examination was performed after sectioning the heart transversely along the length of the linear monofilament template used for injections. A second, parallel transverse section was made basal to the first to obtain a 5-mm-thick slice that included the left ventricle as well as the septum and right ventricle. This slice was fixed at 4°C for 16 hours in a solution of 1× PBS, 1.25% glutaraldehyde, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 before it was rinsed in PBS and stained for 3.5 hours at 24°C in a buffered solution of the X-gal chromagen [100 mmol/L sodium phosphate, pH 7.3, 1.3 mmol/L $MgCl_2$, 3 mmol/L $K_3Fe(CN)_6$, 3 mmol/L $K_4Fe(CN)_6$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)] as described by MacGregor et al.[20] Prolonged incubation (>8 hours) produced a diffuse background of dark blue that was clearly artifactual.

The myocardial tissue on the distal surface of the section made along the longitudinal line defined by the injection template was processed for histochemical and immunohistochemical analysis. Fresh samples were suspended in tissue-freezing medium and snap-frozen in a bath of isopentane cooled by liquid nitrogen. Cryostat sections (4 μm thick) were collected on Probe-On Plus slides (Fisher Scientific Co) and fixed with 1.25% glutaraldehyde for 10 minutes at 4°C. The tissue sections were rinsed and incubated for 3.5 hours at 24°C in a buffered solution of the X-gal chromagen as described above. After color development, the sections were rinsed again in PBS and subjected to immunohistochemistry as described below or counterstained with nuclear fast red before mounting for photomicroscopy.

Immunohistochemistry

Immunohistochemistry was performed using monoclonal antibodies directed against porcine CD8 (PT36B) and CD44 (BAT31A) (kind gifts of W.C. Davis, Washington State University, Pullman, Wash). On completion of the X-gal staining procedure described above, the sections were rinsed in PBS, treated with normal horse serum, and incubated overnight at 24°C with primary antibody (1 μg/mL). The sections were then incubated with an alkaline phosphatase–conjugated secondary antibody raised against mouse IgG (Biogenex Laboratories). Levamisol (20 μL/mL) was included with the secondary antibody to inhibit endogenous alkaline phosphatase activity. The chromagen, nitro-blue tetrazolium (Sigma Chemical) was deposited at antigenic sites to yield a deep purple reaction product before counterstaining with nuclear fast red.

Systolic Thickening Fraction

		1 d After Surgery	7 d After Surgery
Ad5-injected hearts	Site 1	22.1±7.1	24.1±4.6
	Site 2	25.9±0.9	23.5±3.2
Control hearts	Site 1	22.6±4.0	24.4±5.8
	Site 2	26.5±1.4	20.2±1.9

In each of the six swine, two miniature Doppler ultrasonic crystals were sutured onto the anterior and anterolateral surfaces (site 1 and site 2, respectively) of the heart to assess regional myocardial function as systolic thickening fraction. The two sites of Doppler probe placement in each of the three experimental (Ad5-injected) hearts corresponded to sites of intramyocardial injection of Ad5/RSV/GL2 at doses of 0.7×10^7 and 7.0×10^7 pfu, respectively, whereas the three control hearts did not undergo injection. Values are mean±SEM, n=3 for Ad5-injected hearts, and n=3 for control hearts.

Quantitative Image Analysis

After X-gal histochemistry, slides were reviewed using computer-assisted image analysis to objectively assess the frequency and distribution of cardiac myocytes expressing β-galactosidase. A color videocamera attached to an Olympus VANOX microscope ported images to a 24-bit true color frame grabber. The captured images were then relayed to the Optimas imaging system for analysis. Microscopic magnification was set at 134× to display a total myocardial area of 0.03 mm^2 on the video monitor for analysis. Briefly, the field exhibiting the greatest staining intensity was chosen using area gray scale value as the determinant. This field was designated as the origin from which four radii were examined at 45°, 135°, 225°, and 315° relative to the displayed video image. In addition to the central 0.03-mm^2 field, nine contiguous fields were sampled along each radius to a distance of 2.375 mm from the origin, each field being defined by calibrated field dimensions of 0.195×0.152 mm. The percentage of β-galactosidase–positive cardiomyocytes per field was then calculated from the total numbers of blue-stained (positive) and unstained (negative) cardiomyocytes in each field.

Statistical Analysis

All values are reported as mean±SEM. Measurements of luciferase activity were analyzed by nonparametric methods (Kruskal-Wallis test and Wilcoxon signed rank test) because the data did not follow a normal distribution.[29]

Results

Effect of Ad5 Injection on Regional Myocardial Function

To assess the impact of Ad5 injection on regional myocardial function, pulsed Doppler probes were sutured onto sites of Ad5/RSV/GL2 injection in three of the experimental swine, and systolic thickening fraction was measured as previously described.[28,30] The values of thickening fraction at serial times after injection were compared with those obtained from three control swine that underwent a left thoracotomy and Doppler probe implantation but were not subject to intramyocardial injection. Systolic thickening fraction was found to be similar in the two groups at all time points (Table). Thus, the trauma associated with intramyocardial injection of adenovirus, the expression of foreign genes, and the immunological response of the host did not have a measurable effect on regional myocardial function as assessed by this method. Furthermore, there were no appreciable differences between the two groups with respect to heart rate or arterial pressure (data not shown).

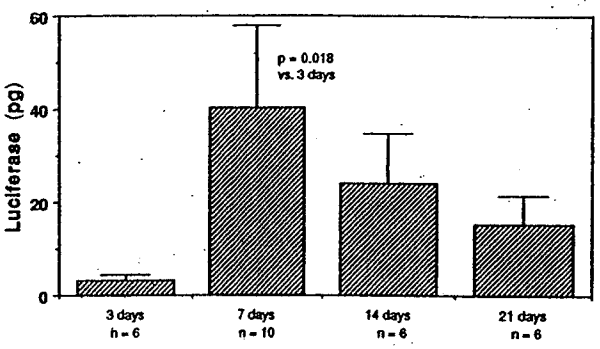


FIG 1. Bar graph of time course of Ad5-mediated gene expression after direct injection into porcine myocardium. A 100-μL volume of phosphate-buffered saline containing 7×10^7 pfu of a recombinant adenovirus carrying the luciferase reporter gene (Ad5/RSV/GL2) was injected into the left ventricular wall of open-chested pigs. The animals were killed while under deep anesthesia 3, 7, 14, and 21 days after the procedure, and transmural samples encompassing the injection sites were homogenized for determination of luciferase activity. Each column represents the mean amount of luciferase recovered from 6 to 10 injection sites as indicated by n on the bottom row. Values are mean±SEM.

Time Course of Ad5-Mediated Gene Expression After Direct Injection

To determine the magnitude and duration of Ad5-mediated gene expression, the swine were euthanized at 3, 7, 14, and 21 days after Ad5 injection for luciferase assay. Fig 1 illustrates the time course of luciferase expression following the injection of 7×10^7 pfu of Ad5/RSV/GL2 into the left ventricular wall. Luciferase activity was detectable as early as 3 days after injection but increased markedly at 7 days and then declined progressively at 14 and 21 days. Statistical analysis (nonparametric one-way ANOVA with the Kruskal-Wallis test) demonstrated that there was a significant ($P=.018$) increase in luciferase activity between 3 and 7 days. As maximal activity was obtained at 7 days, this time point was chosen for the experiments summarized below.

Dose-Response Relation Using a Cell Lysate Preparation

To characterize the relation between the amount of virus injected and the amount of recombinant protein produced, increasing doses of Ad5/RSV/GL2 (from 0.7×10^7 to 1.8×10^8 pfu) were injected into the left ventricular wall and luciferase activity was measured 7 days later. As shown in Fig 2, as little as 0.7×10^7 pfu produced detectable amounts of luciferase (1.1±0.7 pg). With increasing amounts of Ad5 virus, the production of luciferase increased in a dose-dependent manner, reaching 120±47 pg at a dose of 1.8×10^8 pfu.

Comparison of Ad5 With Plasmid DNA

The efficiency of Ad5- versus plasmid-mediated gene transfer was compared using a reporter plasmid (pRSVL),[27] which carries a luciferase expression cassette analogous to the one in Ad5/RSV/GL2. Using identical injection volumes of 100 μL and the same PBS

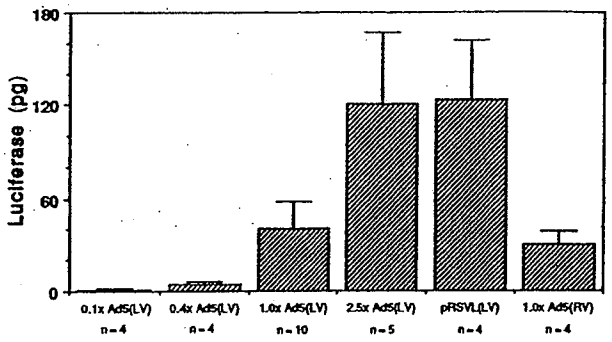


FIG 2. Bar graph of dose-response relation for Ad5/RSV/GL2, comparison with plasmid DNA, and comparison with right ventricular injection. The effect of varying the administered dose of virus was investigated by injecting into the left ventricle (LV) 100-μL volumes of phosphate-buffered saline (PBS) containing 0.7×10^7 pfu (0.1× Ad5), 2.8×10^7 pfu (0.4× Ad5), 7×10^7 pfu (1.0× Ad5), and 1.8×10^8 pfu (2.5× Ad5) of Ad5/RSV/GL2 and determining the amount of luciferase produced at each injection site after 7 days. Injections containing 200 μg of a plasmid DNA bearing an analogous expression cassette (pRSVL) in 100 μL of PBS were made into the left ventricle, and luciferase production was measured after 7 days. To determine whether the efficiency of Ad5-mediated gene transfer was constant in the left and right ventricles, 7×10^7 pfu of Ad5/RSV/GL2 in 100-μL volumes of PBS were injected into the right ventricle (RV), and luciferase production was determined after 7 days. Each column represents the mean amount of luciferase recovered from 4 to 10 injection sites as indicated by n on the bottom row. Values are mean±SEM.

vehicle used to deliver the Ad5 reporter virus, 200-μg quantities of pRSVL DNA were directly injected into the left ventricular wall. This quantity of plasmid DNA was chosen because it had previously been shown to yield maximal reporter gene expression following direct left ventricular injection in dogs.[8] As shown in Fig 2, the mean luciferase activity produced by the luciferase reporter DNA was similar to that produced by 1.8×10^8 pfu of the luciferase reporter virus.

Ad5-Mediated Gene Expression in the Right Ventricular Wall

It is unknown whether Ad5 vectors can be used to transfer genes into the right ventricle and how their efficiency in this location compares to that in the left ventricle. Because of the small heart size, elucidation of this issue in rodents would be difficult. Accordingly, 7×10^7 pfu of Ad5/RSV/GL2 were injected into the right ventricular wall and luciferase activity was measured 7 days later. As depicted in Fig 2, the amount of luciferase produced was comparable to that measured following similar injections into various regions of the left ventricle. Thus, the Ad5 reporter virus appears to be equally efficient in gene transfer to the right and left ventricles.

High-Level Expression Using a Purified Preparation

The highest dose of recombinant Ad5 presented in Fig 2 approaches the maximum that is possible using a cell lysate preparation; however, step and density gradient centrifugation procedures can be used to further concentrate and purify Ad5 by several orders of magnitude. To achieve higher levels of expression, a cell lysate preparation of Ad5/RSV/GL2 was subjected to two rounds of CsCl centrifugation and dialyzed against 1× PBS, 1% sucrose to produce a working stock with a

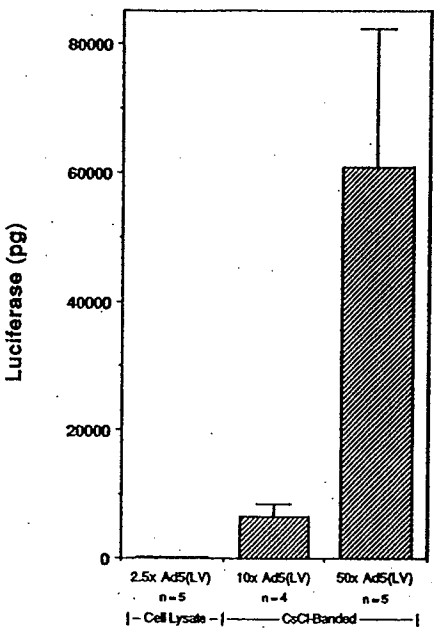


FIG 3. Bar graph of luciferase production from concentrated Ad5/RSV/GL2. To produce higher levels of luciferase, a cell-lysate preparation of Ad5/RSV/GL2 was purified by two rounds of CsCl centrifugation and dialyzed against 1× phosphate-buffered saline (PBS), 1% sucrose to yield a purified preparation with a concentration of 4×10^{10} pfu/mL. Dilutions of this preparation were made in vehicle (1× PBS, 1% sucrose) to contain either 7×10^8 (10× Ad5) or 3.6×10^9 pfu (50× Ad5) in each of the 100-μL volumes injected into the left ventricle. The resulting levels of luciferase are compared with the level resulting from the highest dose of cell lysate preparation tested in Fig 2 (2.5× Ad5). Each column represents the mean amount of luciferase recovered from four or five injection sites as indicated by n below the relevant column. Values are mean±SEM.

concentration of 4×10^{10} pfu as determined by plaque assay. Fig 3 compares the results of direct myocardial injections using dilutions of this concentrated preparation with the highest dose of cell lysate preparation, which was presented in Fig 2. The increased input of Ad5/RSV/GL2 produced higher levels of luciferase, and the absence of a plateau in the dose-response relation suggests that the kinetics of viral infection and gene expression were not saturated even at the highest dose of recombinant Ad5 examined.

Distribution of Gene Expression

To determine the identity of the cells targeted by Ad5 vectors and to characterize their distribution in the myocardium, histochemical analyses were performed on tissue samples obtained 7 days after intramyocardial injection with CsCl-purified preparations of a replication-deficient Ad5 vector carrying the *E. coli lacZ* reporter gene (Ad5/HCMV/*LacZ*). The β-galactosidase produced from this vector was detected in gross tissue and cryostat sections by histochemical staining with a chromagenic substrate (X-gal) that yields an insoluble indigo reaction product in the presence of enzyme. Fig 4B depicts a porcine heart that was sectioned transversely along a 2.5-cm line of 10 (100-μL) injections, each delivering 1.4×10^9 pfu of Ad5/HCMV/*LacZ*. A 2.5-cm line of negative control injections consisting of vehicle alone (1× PBS, 1% sucrose) was also included in this section on the lateral wall of the left ventricle,

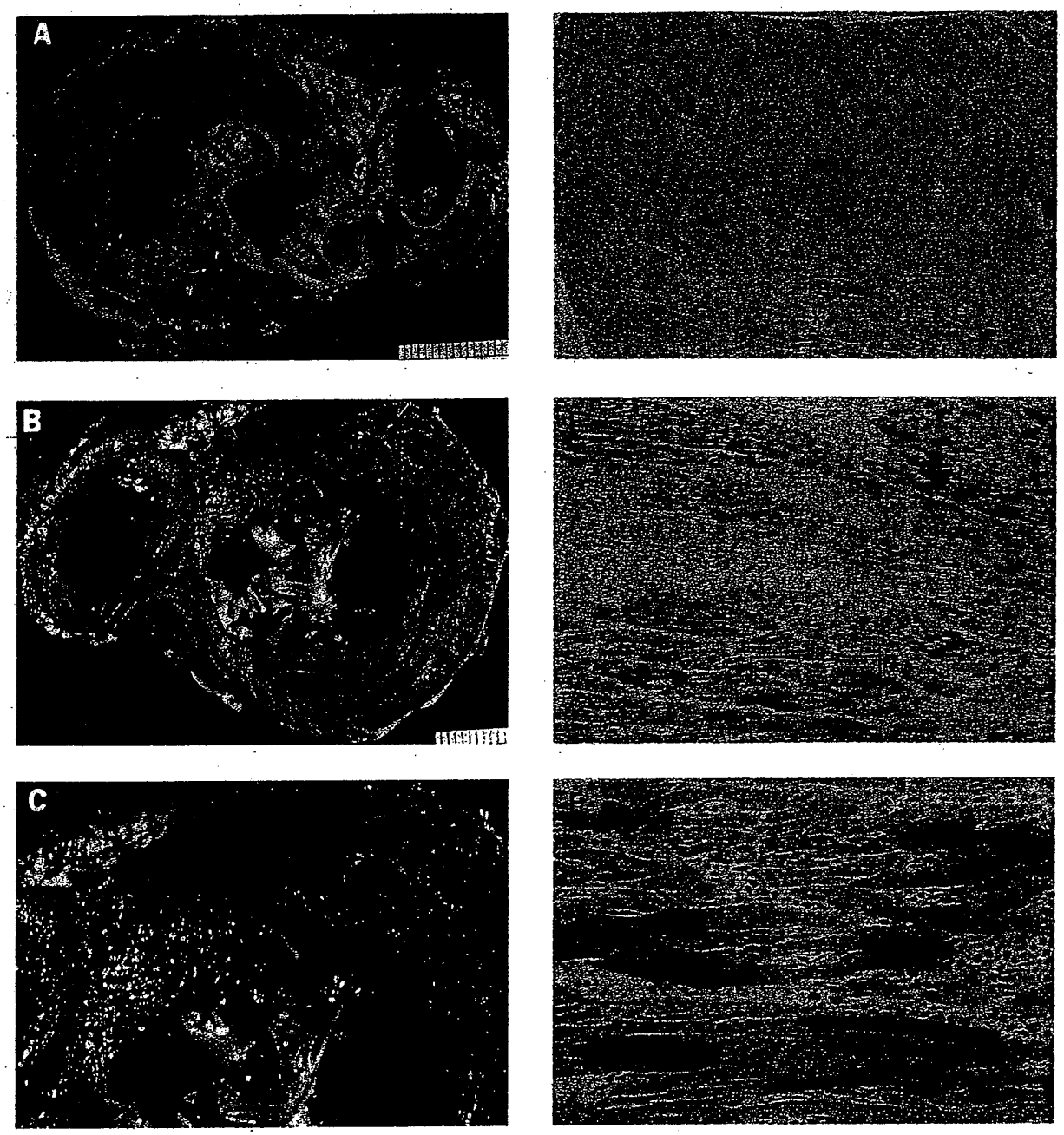


FIG 4. Photomicrographs of distribution of Ad5-mediated gene transfer as determined by histochemical assay for β-galactosidase activity. A recombinant adenovirus carrying the *lacZ* reporter gene (Ad5/HCMV/*LacZ*, kindly provided by A. Bett and F.L. Graham) was purified to a concentration of 1.4×10^{10} pfu/mL and used to make 10 direct injections along the anterior wall of the left ventricle. A linear monofilament template 5 cm long was sutured onto the left ventricular epicardium in a plane parallel to the atrioventricular groove, beginning at the level of the left anterior descending artery and ending on the obtuse margin. A series of 10 injections, each containing 1.4×10^{9} pfu of reporter virus, was made along the 2.5-cm anterior half of this template at 2.5-mm intervals, while a series of 10 injections of vehicle (1× phosphate-buffered saline [PBS], 1% sucrose) was made along the lateral half. Seven days after direct injection, the animal was killed, and the heart was sectioned transversely along the line defined by the injection template. B and C, Proximal surface of this incision; D through F, distal surface of the incision. A second section was made parallel and 5 mm proximal to the first to generate the slice illustrated in A through C. This slice was fixed in 1× PBS, 1.25% glutaraldehyde, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 for 16 hours, rinsed in PBS, and then stained for 3.5 hours in a solution of X-gal substrate. A demonstrates that no gross evidence of β-galactosidase activity could be found on the proximal aspect of the slice (5 mm proximal to the line of injections). In contrast, B shows that considerable activity was evident along the 2.5-cm line of Ad5 injections in the anterior wall of the left ventricle (between the arrowheads at left and center). The total lack of blue staining along the 2.5-cm line of negative control injections in the lateral wall (between the arrowheads at center and right) demonstrates the specificity of the histochemical assay. Prolonged staining (>8 hours) resulted in a diffuse darkening of the entire sample, which obscured the true positive signal (not shown). As illustrated in C, close examination of the positive region after 3.5 hours of incubation revealed irregularities in the distribution of the blue stain. D through F, Photomicrographs of frozen sections obtained from the distal surface of the incision made along the injection template. The low-power (5×) magnification in D shows that the β-galactosidase–positive (blue) cells were grouped in loose clusters surrounding areas of pronounced cellular infiltrate (indicated by asterisks). The bracketed region in D is enlarged to a 20× original magnification in E to show the border between the cellular infiltrate and the Ad5-infected cardiomyocytes. The bracketed region in E is enlarged to an 80× original magnification in F to reveal the morphology of the positively stained cardiomyocytes and their association with the cellular infiltrate.

immediately adjacent to the viral injections. After fixation and incubation with the X-gal chromagen, an area of intense blue staining (approximately 2.5 cm long and 0.7 cm wide). No evidence of X-gal staining was found in the adjacent 2.5-cm-long segment of left ventricle that was injected with vehicle alone. A second, parallel section was made 5 mm basal to the first to generate the gross specimen. Fig 4A demonstrates that there was no evidence of staining on the basal aspect of the slice, at a distance of 5 mm from the series of 10 viral injections. Transverse sections made after photography revealed that the gross positive staining shown in Fig 4B was quite superficial, extending no more than five cell layers in depth. However, subsequent X-gal staining and microscopic analysis of these transverse sections demonstrated that a few positive cardiomyocytes could be found up to 5 mm away from the line of viral injections (data not shown). This indicates that many β-galactosidase–positive cells in the deeper layers of the gross specimen failed to stain blue on the initial immersion in X-gal, presumably due to limited penetration by the chromagenic substrate.

Although macroscopic examination (Fig 4B and 4C) suggested the presence of extensive regions of high-frequency gene transfer, the photomicroscopy in Fig 4D (5× original magnification) demonstrates that positive (blue) cells were actually grouped in loose clusters adjacent to the site of injection. The bracketed region in Fig 4D is enlarged in Fig 4E (20× original magnification) to illustrate that positive cardiomyocytes were associated with regions of increased cellularity. The dense cluster of positive cells in Fig 4E is enlarged in Fig 4F (80× original magnification) to illustrate the morphological features of the recombinant cardiomyocytes and the cellular infiltrate. Careful analysis at high magnification revealed that nearly all (>95%) of the clearly definable cells that stained positive with X-gal also contained the striations characteristic of cardiomyocytes. When β-galactosidase–positive clusters were examined in high-power fields containing 60 to 70 cardiomyocytes, up to 75% of the cardiomyocytes showed evidence of the blue stain. However, the computer-assisted image analysis summarized in Fig 5 revealed that the percentage of blue cardiomyocytes was found to decrease markedly as a function of distance from the most positive microscopic field.

Immunohistochemical Characterization of Leukocytic Infiltrate

Analysis of the histochemical sections presented in Fig 4 revealed that the cardiomyocytes that stained blue in the X-gal assay for β-galactosidase activity were often associated with infiltrating populations of small, spherical cells containing prominent nuclei. Furthermore, cell-mediated cytolysis was suggested by the observation that the blue cardiomyocytes surrounded by infiltrating cells often exhibited features of cellular deterioration. To further characterize this phenomenon, cryosections that had undergone the X-gal staining procedure were additionally subjected to immunohistochemistry using monoclonal antibodies developed against porcine CD44 and CD8. An immunohistochemical technique using alkaline phosphatase as the reporter system was used to label antigenic sites with nitro-blue tetrazolium to yield a deep purple reaction product. Tissue sections in Fig

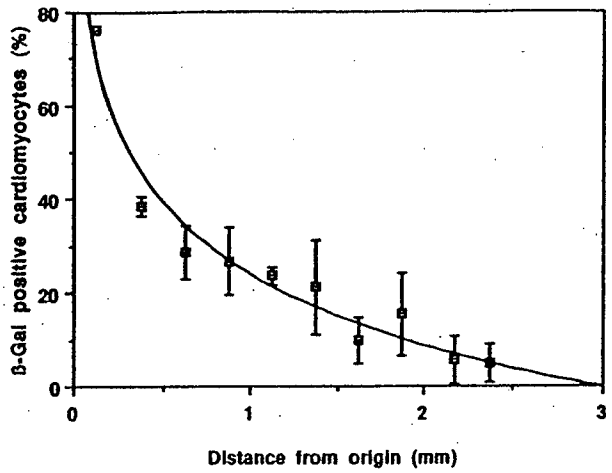


FIG 5. Plot of quantitative image analysis of β-galactosidase distribution. Computer-assisted quantitative image analysis was performed on a representative X-gal–stained frozen section from the injected region of myocardium as described in "Methods." The percentage of cardiomyocytes staining blue in the X-gal assay for β-galactosidase activity was determined in nine consecutive fields along four lines originating from a highly positive, rectangular field with dimensions of 0.152×0.195 mm. The mean percentages of β-galactosidase (β-gal)–positive cardiomyocytes are plotted on the *y* axis with error bars indicating SEM. The distance from the center of the highly positive field to the most distal point examined in each 0.152×0.195-mm field is plotted on the *x* axis.

6A through 6C were stained by X-gal and the CD44 antibody, and sections in Fig 6D through 6F were stained with X-gal and the CD8 antibody. Fig 6A demonstrates that the injection of vehicle alone resulted in a small, well-defined region of leukocytic infiltration (indicated by the CD44 antigenic sites labeled deep purple). The presence of the CD44 antigen (lymphocyte homing receptor) on the membranes of the infiltrating cells is consistent with a normal healing response to intramyocardial injection (20×). Fig 6D depicts a similar vehicle-injected region in which few T cells were detected by immunohistochemistry using the monoclonal antibody developed against CD8 (20×). Fig 6B demonstrates that the injection of Ad5/HCMV/*LacZ* induced a far more prominent inflammatory response that was centered on the Ad5-infected cardiomyocytes identified by the X-gal stain (20×). Fig 6E shows a similar site of Ad5/HCMV/*LacZ* injection immunostained with the CD8 antibody (20×). The injection of Ad5 clearly stimulated myocardial infiltration by the small, spherical CD8-positive cells. The bracketed region in Fig 6B is enlarged in Fig 6C (80×) to illustrate several examples in which CD44-positive cells were associated with the cytolysis of infected cardiomyocytes. Arrowheads indicate blue-stained cardiomyocytes in advanced states of cytolysis. The bracketed region in Fig 6E is enlarged in Fig 6F (80×) to illustrate that members of the CD8-positive population were less intimately involved in the lytic process.

Discussion

The salient findings of this study can be summarized as follows. (1) Replication-deficient Ad5 vectors are capable of mediating recombinant gene expression after direct injection into adult porcine myocardium. (2) The

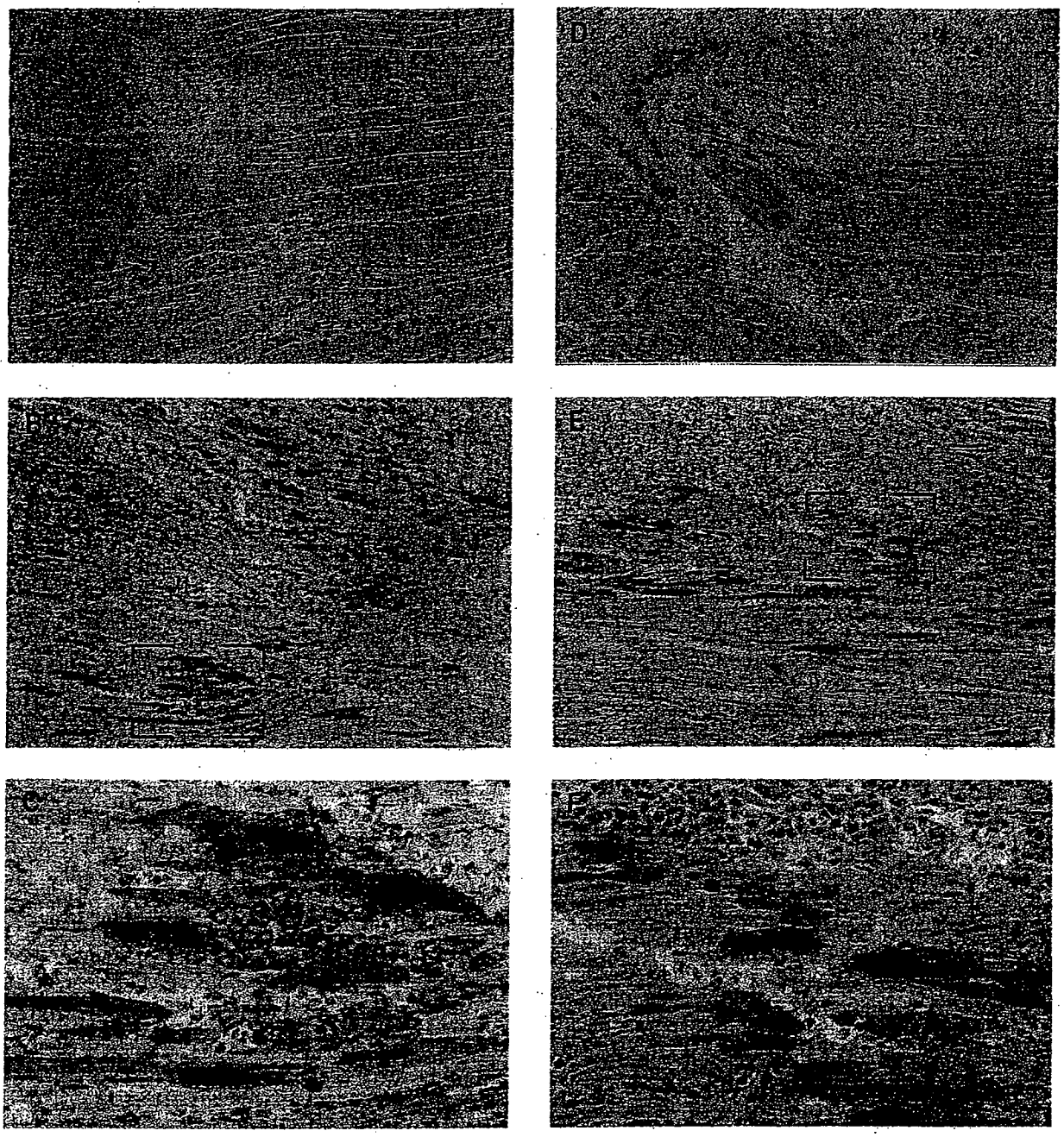


FIG 6. Photomicrographs of immunohistochemical assessment of leukocytic infiltration. X-gal–stained frozen sections from the distal surface of the incision made along the series of negative control injections (A and D) or along the series of Ad5/HCMV/*LacZ* injections (B, C, E, and F) were additionally subjected to immunohistochemistry using monoclonal antibodies developed against porcine CD44 (A, B, and C) or CD8 (D, E, and F). A (20× original magnification) shows that modest populations of CD44-positive leukocytes (labeled deep purple by nitro-blue tetrazolium) could be found at sites of vehicle injection. B (20×) shows that penetration of the myocardial region by the CD44-positive leukocytes was significantly increased when the 100-μL injection contained 1.4×10^9 pfu of recombinant Ad5. The bracketed region in B is enlarged to 80× in C to demonstrate that the CD44 leukocytes are associated with the degradation of Ad5-infected (blue) cardiomyocytes. Arrowheads indicate examples of cardiomyocytes in advanced states of cytolysis. D (20×) shows that few CD8-positive T cells (labeled deep purple by nitro-blue tetrazolium) could be found at sites of vehicle injection. E (20×) shows that the population of these small, spherical cells was markedly increased when the injection delivered recombinant Ad5. The arrowhead in F (80×) indicates a representative CD8-positive T cell. Although T cells could be found near the Ad5-infected cardiomyocytes, they were less intimately associated with the degradation of these cells than the CD44-positive leukocytes (compare with C).

impact of this procedure on regional cardiac function appears to be negligible. (3) Luciferase expression peaks around 7 days and persists for at least 3 weeks. (4) The amount of recombinant protein increases with the amount of virus, with no evidence for a plateau, as the viral dose is increased ~500-fold from 0.7×10^7 to 3.6×10^9 pfu. (5) On a molar basis, the Ad5 vector is 140 000 times more efficient than plasmid DNA in mediating gene transfer. (6) The expression of recombinant genes following intramyocardial injection of Ad5 is similar in the left and right ventricles. (7) Such expression specifically involves cardiomyocytes. (8) The percentage of cardiomyocytes expressing β-galactosidase can be high in microscopic regions adjacent to the

injection site (up to 75% in fields of 60 to 70 cells). (9) Essentially no luciferase activity (and very few β-galactosidase–positive cells) can be detected farther than 5 mm from the injection site. (10) And the recombinant Ad5 virus carrying the β-galactosidase reporter gene induces a prominent inflammatory response. Previous studies have shown that gene transfer into the heart can be achieved with intramyocardial injection of plasmid DNA in rats[4-7] or dogs.[8] The myocardium has also been targeted with the intravenous injection of Ad5 vectors in mice,[11] with transcatheter infusion of Ad5 vectors into the coronary ostia of rabbits,[19] and with direct injection of Ad5 vectors in rat hearts.[16] However, to our knowledge, this is the first study in which Ad5 vectors have been used to mediate direct gene transfer into the heart of a large mammalian species.

The importance of using large animals such as swine stems from the fact that rodents differ substantially from humans in many aspects of cardiovascular physiology and pathophysiology, including rate of metabolism, pattern of cardiac contractile isoforms,[31,32] and induction of isoform switching during development and hypertrophy.[33,34] Moreover, the porcine model makes possible sophisticated measurements of cardiac function and flow in longitudinal studies at serial time points, which would be extremely difficult in rats or mice. Thus, gene transfer into porcine hearts may be useful for analyzing the regulation of gene expression in a species that is more relevant to humans than rodents. Furthermore, this approach provides a model in which cardiac physiology can be accurately monitored over extended periods of time.

The results of the measurements of wall thickening (Table) indicate that as many as 16 separate low-dose injections of the luciferase reporter virus did not adversely impact regional myocardial function. This suggests that the porcine heart can be used for multiple low-dose intramyocardial injections of recombinant Ad5 without significantly interfering with cardiac contractility. A limitation of these data, however, is that ultrasonic crystals were not used in the experiments in which the more concentrated, CsCl-banded preparations of recombinant Ad5 were injected and pronounced inflammation was documented. Furthermore, the relatively small regions monitored by the ultrasonic crystals may not have corresponded exactly to the sites of Ad5 injection. Accordingly, the results presented in the Table do not formally exclude the possibility that high doses of recombinant Ad5 might impair contractility in the immediate vicinity of the injection site.

The data presented in Fig 1 indicate that recombinant gene expression persisted for at least 21 days, with a peak at approximately 7 days after gene transfer. This temporal pattern of gene expression is similar to that observed by Buttrick et al[7] and von Harsdorf et al[8] following the direct injection of plasmid DNA into rat and canine myocardium, respectively. The concordance of these results is consistent with the episomal location of both plasmid and adenoviral DNAs in the nuclei of transfected (or infected) cells. These extrachromosomal DNAs are subject to similar nuclear processes that might be expected to reduce the levels of recombinant gene expression at later time points. However, the immunohistochemistry presented in Fig 6 strongly suggests that an inflammatory response involving cell-mediated cytolysis may play a critical role in the decline of Ad5-mediated reporter gene expression that we observed starting approximately 7 days after direct injection (Fig 1).

The persistence of expression following direct gene transfer into the myocardium has been reported to vary considerably.[5,11] Neonatal mice injected intravenously with a replication-deficient adenovirus maintained sustained levels of β-galactosidase in many tissues (including skeletal muscle and heart) for a period of 10 months, although similar injections in adult mice resulted in a much shorter time course of reporter gene expression.[11] A mechanism for the extinction of reporter gene expression involving cell-mediated cytolysis could explain this apparent discrepancy, since the injection of recombinant Ad5 into the immunologically naive neonatal mice probably induced a state of immune tolerance. The use of cyclosporin A or FK-506 to suppress the natural inflammatory response of adult animals to viral infection would therefore be expected to potentiate and prolong Ad5-mediated gene expression following direct in vivo gene transfer. Nevertheless, it is reasonable to expect a gradual decrease in the levels of recombinant gene expression from episomal DNAs due to cellular mechanisms such as DNA methylation or nuclease action. For example, Acsadi et al[5] have reported that luciferase expression resulting from direct injection of plasmid DNA into adult rat heart was reduced approximately 500-fold by 25 days. Therefore, species- or age-related variables, the nature of the gene transfer system and protein product, immunological interdiction, as well as factors controlling DNA stability, promoter utilization, messenger RNA stability, and protein degradation may all play important roles in determining the duration of recombinant gene expression following direct in vivo gene transfer.

One of the major findings of this study was that the infection of cardiomyocytes with high doses of Ad5 was associated with a marked leukocytic infiltration, which presumably limited the intensity and duration of recombinant gene expression and caused notable tissue damage (Fig 6). This inflammatory response is consistent with previous studies showing that although the deletion of the E1 genes is adequate to render Ad5 vectors replication deficient, it does not completely silence the remainder of the viral genome.[35,36] It would appear that the presentation of expressed viral proteins on the surface of infected cells induces an inflammatory response that involves cytotoxic T-lymphocytes, monocytes-macrophages, and/or natural killer cells. Similar leukocytic infiltrates have been characterized in various tissues after Ad5-mediated direct in vivo gene transfer in several animal models.[37,38] Efforts are presently under way to obviate this problem. In particular, recent work has demonstrated that additional modifications to the adenoviral genome can reduce the immune response, presumably by attenuating viral gene expression.[39,40] These modifications should result in the production of higher levels of recombinant protein for longer periods of time following direct intramyocardial injection than was demonstrated here.

In the present study, similar levels of gene expression were obtained in the right and left ventricles after direct injection of the luciferase reporter virus. This contrasts with the results of von Harsdorf et al,[8] in which direct injection of plasmid DNA resulted in approximately threefold more reporter protein in the left ventricle

than in the right ventricle. It is possible that this difference is due to the oblique injection angle used here to prevent the needle from penetrating the relatively thin right ventricular wall. The lack of regional differences in the levels of gene expression between the left and right ventricles makes the porcine model suitable for comparison of several different viral vectors within one animal, thereby eliminating interindividual variability. This represents an advantage over studies in rodents, in which large numbers of animals are required to achieve statistical significance.[35]

To our knowledge, this is the first study to accurately quantitate the production of recombinant protein following either Ad5- or plasmid-mediated direct gene transfer to the heart. As demonstrated in Figs 2 and 3, there exists a positive relation between the amount of replication-deficient Ad5 vector injected and the levels of recombinant protein produced. The highest concentration of virus used in Fig 2 (1.8×10^9 pfu/mL) approaches the maximum that can be obtained from a cell lysate preparation, so CsCl centrifugation was used to produce a stock of Ad5/RSV/GL2 with a plaque assay titer of 4×10^{10} pfu/mL. Experiments using this purified preparation demonstrated that a single 100-μL injection containing 3.6×10^9 pfu of reporter virus produced 61±21 ng of luciferase. The fact that this high dose resulted in increased levels of recombinant protein suggests that the myocardial capacity for recombinant gene expression was not saturated under the conditions of this study. Given the very short half-life of luciferase in mammalian cells, it is conceivable that a more stable recombinant gene product would accumulate to even higher levels.

The experiments summarized in this report were not designed to provide a comprehensive comparison between Ad5- and plasmid-mediated gene transfer. Nevertheless, Figs 2 and 3 suggest that Ad5/RSV/GL2 offers a significant advantage over plasmid DNA. The highest dose of reporter virus examined in the present study (3.6×10^9 pfu) produced luciferase levels that were more than 500-fold higher than those obtained from the direct injection of 200 μg of plasmid DNA. This particular dose of plasmid DNA was chosen because it yielded maximal levels of reporter gene expression when injected into the left ventricle of intact dogs using techniques similar to those used in this study.[8] The expression levels obtained with the pRSVL plasmid DNA in our study may therefore approach the maximum possible, whereas the results obtained with Ad5 might still be improved by 5- to 10-fold with more sophisticated concentration protocols. Furthermore, it is important to consider gene transfer efficiency in terms of the number of recombinant genes injected. In this study, 200 μg of pRSVL DNA (42.4 pmol of reporter gene) produced a mean of 123 pg of luciferase per injection site, whereas 1.8×10^8 Ad5 particles (0.30 fmol of reporter gene) produced comparable levels. Therefore, when considered on a molar basis, the Ad5 is 140 000 times more efficient than plasmid DNA in mediating gene transfer in vivo after direct intramyocardial injection.

The quantitative measurements of luciferase activity did not allow us to discern whether the recombinant protein was expressed in myocytes or in other cell types such as endothelial cells, fibroblasts, or even the inflammatory cells that infiltrate the needle track after intramyocardial injection.[4,5] This is an important issue if one wishes to target a specific cell population for the purposes of gene therapy. Therefore, to assess the cellular distribution of recombinant gene expression and to determine if it occurred specifically in cardiomyocytes, a replication-deficient virus carrying the *lacZ* reporter gene was injected into the left ventricle and infected cells were identified by histochemical staining of serial sections harvested 7 days later. On histological evaluation, the vast majority (>95%) of the blue-stained cells could be identified as cardiomyocytes by their characteristic pattern of striations (Fig 4). Thus, the β-galactosidase reporter gene is selectively expressed in cardiomyocytes after direct intramyocardial injection with replication-deficient Ad5 as has been reported after direct DNA injection.[4,5] This is in contrast to the cellular distribution of reporter gene expression observed after administration of Ad5 via intravenous injection (which targets a wide range of tissues[11]) or via transcatheter infusion to the coronary sinus (which targets both the vasculature and the myocardium[19]).

Gross examination of ventricular slices after histochemical staining for β-galactosidase activity (Fig 4) revealed macroscopic evidence of recombinant gene expression. The authenticity of the positive signal was demonstrated by the total lack of staining in the lateral ventricular region injected with vehicle alone. Although the macroscopic assessment was visually impressive, microscopic examination of X-gal–stained cryosections revealed a heterogeneous distribution of infected cells in loose clusters near the injection sites. The percentage of infected cells was high (up to 75%) at the center of these microscopic clusters but decreased markedly as a function of distance as illustrated in Fig 5. It would therefore appear that Ad5 vectors have a limited capacity for migration through adult porcine myocardium after direct intramyocardial injection. Accordingly, it is unlikely that a single direct injection of replication-deficient Ad5 virus (or of plasmid DNA[7]) will genetically alter significant regions of the myocardium in a uniform manner. More widespread gene expression can be obtained by making multiple, contiguous injections as demonstrated in Fig 4, but this does not appear to be a practical method for modulating gene expression over large regions of myocardium. These observations have implications for the application of somatic cell gene therapy in the heart, since therapeutic strategies involving the secretion of recombinant proteins (eg, vasoactive peptides or angiogenic growth factors) from a subset of cardiomyocytes may be more successful than those that rely on uniform gene transfer throughout large portions of the myocardium.

In summary, the present study demonstrates that direct intramyocardial injection of replication-deficient Ad5 vectors is a feasible and practical method for introducing recombinant genes into the cardiomyocytes of a large animal species with relevance to human physiology. The efficiency of Ad5-mediated gene transfer was superior to that of plasmid DNA injection in this study, and replication-deficient Ad5 vectors offer the advantage of producing more recombinant protein. Successful genetic modulation of discrete myocardial regions with Ad5 vectors could find application in studies of in vivo gene expression regulation in response to stimuli that cannot be mimicked in vitro. However, the in vivo application of these vectors appears to be limited by marked leukocytic infiltration,

probably in response to the residual expression of viral genes by infected cells. Additional recombinant modifications to the Ad5 genome are feasible, which should minimize the inflammatory response and consequently produce higher levels of recombinant gene expression for more extended periods of time.

Acknowledgments

This research was supported in part by Mallinckrodt Medical, Inc (B.A.F.), American Heart Association grant 92012700 (B.A.F.), National Institutes of Health grant HL-43151 (R.B.), and SCOR grant HL-42267 (R.B.). We wish to thank Frank L. Graham and Andrew Bett for generously providing Ad5/HCMV/*LacZ* and the Ad5 cloning system used to generate Ad5/RSV/GL2. We also thank Jennifer S. Pocius, Maria Stanislawska, Jian-Zhong Sun, Xian-Lian Tang, Shufang Tian, and Alejandro L. Tumang for expert assistance in the laboratory.

References

1. Anderson WF. Human gene therapy. *Science (Wash DC).* 1992; 256:808-813.
2. Miller AD. Human gene therapy comes of age. *Nature (Lond).* 1992;357:455-460.
3. Schneider MD, French BA. The advent of adenovirus: gene therapy for cardiovascular disease. *Circulation.* 1993;88:1937-1942.
4. Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. *Circulation.* 1990;82:2217-2221.
5. Acsadi G, Jiao S, Jani A, Duke D, Wulliams P, Chong W, Wolff JA. Direct gene transfer and expression into rat heart in vivo. *New Biol.* 1991;3:71-81.
6. Kitsis RN, Buttrick PM, McNally EM, Kaplan ML, Leinwand LA. Hormonal modulation of a gene injected into rat heart in vivo. *Proc Natl Acad Sci U S A.* 1991;88:4138-4142.
7. Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA. Behavior of genes directly injected into the rat heart in vivo. *Circ Res.* 1992;70:193-198.
8. von Harsdorf R, Schott RJ, Shen Y-T, Vatner SF, Mahdavi V, Nadal-Ginard B. Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals. *Circ Res.* 1993;72:688-695.
9. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. *Science (Wash DC).* 1990;247:1465-1468.
10. Chapman GD, Lim CS, Gammon RS, Culp SC, Desper JS, Bauman RP, Swain JL, Stack RS. Gene transfer into coronary arteries of intact animals with a percutaneous balloon catheter. *Circ Res.* 1992;71:27-33.
11. Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. *J Clin Invest.* 1992;90:626-630.
12. Zabner J, Couture LA, Gregory RJ, Graham SM, Smith AE, Welsh MJ. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *Cell.* 1993;75:207-216.
13. Salle GL, Robert JJ, Berrard S, Ridoux V, Stratford-Perricaudet LD, Perricaudet M, Mallet J. An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* (Wash DC). 1993;259: 988-990.
14. Lemarchand P, Jones M, Yamada I, Crystal RG. In vivo gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. *Circ Res.* 1993;72:1132-1138.
15. Mittal SK, McDermott MR, Johnson DC, Prevec L, Graham FL. Monitoring foreign expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. *Virus Res.* 1993;28:67-90.
16. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. *Circ Res.* 1993;73:1202-1207.
17. Marsh M, Helenius A. Virus entry in animal cells. In: Maramorosch K, Murphy FA, Shatkin AJ, eds. *Advances in Virus Research, Vol 36.* New York, NY: Academic Press; 1989:107-151.
18. Herz H, Gerard RD. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc Natl Acad Sci U S A.* 1993;90:2812-2816.
19. Barr E, Carroll J, Kalynych AM, Tripathy SK, Kozasrsky K, Wilson JM, Leiden JM. Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. *Gene Therapy.* 1994;1:51-58.
20. MacGregor GR, Nolan GP, Fiering S, Roederer M, Herzenberg LA. Use of *E coli lacZ* (β-galactosidase) as a reporter gene. In: Murray EJ, ed. *Methods in Molecular Biology. Vol. 7: Gene Transfer and Expression Protocols.* Clifton, NJ: Humana Press; 1991:217-241.
21. Dannenberg AM, Suga M. Histochemical stains for macrophages in cell smears and tissue sections: β-galactosidase, acid phosphatase, nonspecific esterase, succinic dehydrogenase, and cytochrome oxidase. In: Adams DO, Edelson PJ, Koren MS, eds. *Methods for Studying Mononuclear Phagocytes.* New York, NY: Academic Press; 1981:375-396.
22. Pond WG. Nutrition and the cardiovascular system of swine. In: Stanton HC, Mersmann HJ, eds. *Swine in Cardiovascular Research, Vol. II.* Boca Raton, Fla: CRC Press; 1986:1-31.
23. Wood KV. The origin of beetle luciferase. In: Stanley PE, Kricka LJ, eds. *Bioluminescence and Chemiluminescence.* New York, NY: John Wiley & Sons; 1990:10-14.
24. McGrory WJ, Bautista DS, Graham FL. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology.* 1988;163:614-617.
25. Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ, ed. *Methods in Molecular Biology. Vol. 7: Gene Transfer and Expression Protocols.* Clifton, NJ: Humana Press; 1991:109-128.
26. Morsy MA, Alford EL, Bett A, Graham FL, Caskey CT. Efficient adenoviral-mediated ornithine transcarbamylase expression in deficient mouse and human hepatocytes. *J Clin Invest.* 1993;92: 1580-1586.
27. Keller GA, Gould S, Deluca M, Subramani S. Firefly luciferase is targeted to peroxisomes in mammalian cells. *Proc Natl Acad Sci U S A.* 1987;84:3264-3268.
28. Zhu WX, Myers ML, Hartley CJ, Roberts R, Bolli R. Validation of a single crystal for measurement of transmural and epicardial thickening. *Am J Physiol.* 1986;251:H1045-H1055.
29. Wallenstein S, Zucker CL, Fleiss JL. Some statistical methods useful in circulation research. *Circ Res.* 1980;47:1-9.
30. Bolli R, Patel BS, Jeroudi MO, Lai EK, McCay PB. Demonstration of free radical generation in 'stunned' myocardium of intact dogs with the use of the spin trap alpha-phenyl *N-tert*-butyl nitrone. *J Clin Invest.* 1988;82:476-485.
31. Lompre AM, Mercardier C, Wisnewsky P, Bouveret D, Pantaloni D, D'Albis A, Schwartz K. Species and age dependent changes in relative amounts of cardiac myosin isoenzymes in mammals. *Dev Biol.* 1981;84:286-291.
32. Lompre AM, Nadal-Ginard B, Mahdavi V. Expression of the cardiac ventricular α- and β-myosin heavy chain genes is developmentally and hormonally regulated. *J Biol Chem.* 1984;259: 6437-6446.
33. Lompre AM, Schwartz K, D'Albis A, Lacombe G, Thiem NV, Swynghedauw B. Myosin isoenzyme redistribution in chronic heart overload. *Nature.* 1979;282:105-107.
34. Izumo S, Lompre AM, Matsuoka R, Koren G, Schwartz K, Nadal-Ginard B, Mahdavi V. Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. *J Clin Invest.* 1987;79:970-977.
35. Engelhardt JF, Yang Y, Stratford-Perricaudet LD, Allen ED, Kozarsky K, Perricaudet M, Yankaskas JR, Wilson JM. Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. *Nat Genet.* 1993;4:27-34.
36. Rich DP, Couture LA, Cardoza LM, Guiggio VM, Armentano D, Espino PC, Hehir K, Welsh MJ, Smith AE, Gregory RJ. Development and analysis of recombinant adenoviruses for gene therapy for cystic fibrosis. *Human Gene Therapy.* 1993;4:461-476.
37. Lee SW, Trapnell BC, Rade JJ, Virmani R, Dichek DA. In vivo adenoviral vector-mediated gene transfer into balloon-injured rat carotid arteries. *Circ Res.* 1993;73:797-807.
38. Hertz J, Gerard RD. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc Natl Acad Sci U S A.* 1993;90:2812-2816.
39. Englehardt JF, Ye X, Doranz B, Wilson JM. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc Natl Acad Sci U S A.* 1994;91:6196-6200.
40. Armentano D, Sookdeo C, White G, Giuggio V, Souza D, Couture L, Cardoza L, Vincent K, Wadsworth S, Smith A. Second generation adenovirus vectors for cystic fibrosis gene therapy. *J Cell Biochem.* 1994;(suppl 18A):222. Abstract.

EXHIBIT P

CARDIAC AND PULMONARY REPLACEMENT

CARDIAC GENE TRANSFER BY INTRACORONARY INFUSION OF ADENOVIRUS VECTOR–MEDIATED REPORTER GENE IN THE TRANSPLANTED MOUSE HEART

This study introduces a model for intracoronary gene transfer in murine cardiac isografts using adenovirus vectors. This approach may offer an opportunity to modulate alloreactivity after cardiac transplantation. Donor hearts were infected via the coronary arteries with a volume of 10^9 plaque-forming units per milliliter of a recombinant adenovirus containing the β-galactosidase–encoding gene (Ad.CMVLacZ). In a control group, 200 μl of normal saline solution was infused. The grafts were stored in 4° C cold saline solution for 15 minutes, then transplanted heterotopically into syngeneic hosts (B10.BR). The grafts were harvested at 3, 7, 15, or 30 days ($n = 5$ for each group) after transplantation, and β-galactosidase activity was assessed by histochemical staining (X-gal). All grafts were functioning when harvested. X-gal staining pattern was nonuniform with positive staining appearing in epicardial, myocardial, and endocardial cells, as well as in the vessel walls. The cells permissive to infection consisted predominantly of myocardial cells. The mean total numbers of β-gal–positive staining cells per slice were 68.7 ± 27.3 in the 3-day group, 330.4 ± 53.8 in the 7-day group, 151.3 ± 48.0 in the 15-day group, and 39.9 ± 10.8 in the 30-day group, thus peaking in the 7-day group ($p < 0.05$). Control isografts ($n = 5$), retrieved at day 30, revealed no staining activity. In conclusion, our model demonstrates that intracoronary gene transfer to the transplanted murine cardiac grafts is feasible at the time of harvest. Adenovirus-mediated gene transfer produces widespread gene expression which, though perhaps transient, does not adversely affect myocardial structure or function. This technology may allow modification of graft immunogenicity in the future through the production of therapeutic proteins sufficient to modulate local immune responses. (J THORAC CARDIOVASC SURG 1996;111:246-52)

Jeongryul Lee, MD (by invitation), Hillel Laks, MD, Davis C. Drinkwater, MD, Arie Blitz, MD (by invitation), Lydia Lam, BS (by invitation), Yuji Shiraishi, MD (by invitation), Paul Chang, BS (by invitation), Thomas A. Drake, MD (by invitation), and Abbas Ardehali, MD (by invitation), *Los Angeles, Calif.*

The development of methods to transfer genes into the heart has opened a new era in cardiovascular therapeutics. Through the localized expression of gene products, gene transfer approaches can provide insight into cardiac cell biology and may allow treatment of cardiac diseases such as transplantation coronary artery disease or graft rejection. Several methods of transfecting genes have been studied. In vivo, direct myocardial gene transfer through a needle has been investigated in the rat,[1-5] rabbit, microswine,[6] and dog.[7] However, the limited spatial extent of transfection has restricted the clinical applicability of this technique to human heart diseases.[4] By way of overcoming these limitations, the transvascular approach has been investigated as a potential target for gene therapy because of the

From the Division of Cardiothoracic Surgery, University of California at Los Angeles Medical Center, Los Angeles, Calif.

Read at the Seventy-fifth Annual Meeting of The American Association for Thoracic Surgery, Boston, Mass., April 23-26, 1995.

Address for reprints: Hillel Laks, MD, Division of Cardiothoracic Surgery, UCLA Medical Center, Center for the Health Sciences, Room 62-182A, 10833 LeConte Ave., Los Angeles, CA 90024.

0022-5223/96 $5.00 + 0 12/6/67503

large surface area and widespread distribution. Two types of approaches have been used to transfer genes into endothelial cells in vivo. First, genes have initially been transferred into endothelial cells in vitro, followed by reintroduction of the transfected cells into the vessel wall.[8-10] Second, the genes have been transferred in vivo directly into the vessel wall.[11-13] However, the fact that endothelial cells replicate slowly in the resting state may become a limiting factor for the use of vectors that depend on cell proliferation to express the exogenous genes.[14] A unique feature of adenovirus vectors is that they appear to be efficient in transferring foreign genes even into slowly replicating endothelial cells.[10]

The application of gene transfer techniques to the coronary vessels of cardiac allografts may offer insight into the alteration of alloantigen expression in the grafts and ultimately may allow the treatment of transplantation coronary artery disease or allograft rejection. Early detectable expression of genes after intracoronary infusion of a reporter gene at the time of harvest has been reported.[13]

In the present study, we evaluated the potential utility of adenovirus-mediated cardiac gene transfer by direct infusion of a reporter gene into the coronary arteries of the transplanted mouse heart.

Material and methods

Gene and adenovirus vector. The replication-defective recombinant adenovirus (Ad.CMVLacZ) encoding the *Escherichia coli LacZ* gene, capable of producing the enzyme β-galactosidase, was used as a reporter gene. The gene was modified by the addition of sequences encoding for a nuclear translocation signal and was placed under a control of the cytomegalovirus long terminal repeat. A volume of 200 μl of 10^9 PFU/ml* of viral particles was infused into the donor coronary arteries.

Animals. Fifty adult mice (7 to 10 weeks of age) of the B10.BR strain, weighing 17 to 22 gm, were obtained from Jackson Laboratories (Bar Harbor, Maine). They were housed under conventional conditions and fed a standard diet (Rodent Laboratory Chow, Ralston Purina Company, St. Louis, Mo.) and water. After completion of each procedure, the mice were allowed to recover with oxygen and local heat and transferred to their cages 24 hours after the operation with free access to food.

Heterotopic heart transplantation and intracoronary gene transfer. Cardiac isografts from B10.BR mice were transplanted into a second set of B10.BR mice by means of standard microsurgical techniques. After adequate anesthesia with 4% chloral hydrate (0.1 ml/20 gm of body weight, intraperitoneal injection) and methoxyflurane (inhalation), a sternal lid was lifted upward and fixated. Both the right and the left superior venae cavae were ligated. The donor heart was arrested by infusion of 0.5 ml of cold heparinized saline solution into the inferior vena cava (100 U heparin per milliliter saline solution). The left pulmonary artery was transected to vent the coronary sinus, and the ascending aorta was ligated just proximal to the origin of the innominate artery. The viral particles were then infused into the proximal aorta with a 27-gauge needle. In all cases, outflow of infused solution through the proximal cut end of the left pulmonary artery confirmed adequacy of delivery. The aorta, main pulmonary artery, and the three systemic veins were then transected distal to ligatures, followed by the en bloc ligature and transection of the pulmonary veins. The donor heart was preserved in 4° C normal saline solution during recipient preparation.

Through a midline abdominal incision, the recipient's infrarenal abdominal aorta and inferior vena cava were isolated and ligated both proximally and distally with 5-0 silk. After a longitudinal aortic incision, an end-to-side anastomosis between the donor ascending aorta and the recipient abdominal aorta was performed, followed by an end-to-side anastomosis between the donor pulmonary artery and recipient inferior vena cava. Both anastomoses were sutured with 10-0 nylon. The recipient's proximal aortic ligature was released first so that the graft would be initially reperfused with blood from the proximal aorta.

Expression of β-galactosidase. Histochemical staining was done so that β-galactosidase activity could be examined. Cross sections, 3 to 4 mm in height, were taken from the graft at midventricular level and snap-frozen in liquid nitrogen. Then 10 μm thick slices were cut at 200 μm intervals, followed by fixation with 1.25% glutaraldehyde. After being washed three times at room temperature with phosphate-buffered solution, the slices were stained for β-galactosidase with X-gal (5-bromo-4-chloro-indolyl β-D-galactopyronoside) for 4 to 6 hours, as previously described.[15] Six slices were examined for each mouse. For quantitative analysis of gene expression, the total number of cells positively staining for β-galactosidase was counted per slice under magnification (×40), and a mean value was calculated for six slices. Then, overall mean values were determined for each group (i.e., 3-, 7-, 15-, 30-day, and control groups). With the aforementioned regimen, *LacZ* expression was represented by a nuclear-dominant blue color.

Animal care. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Statistical analysis. Numbers are expressed as mean ± standard deviation of the number observed. The difference of the amount of gene expression was evaluated with analysis of variance. The level of significance was accepted as $p < 0.05$.

Result

Survival. All donor hearts resumed sinus rhythm after several minutes of reperfusion. Total ischemic

*PFU/ml = Plaque-forming unit per milliliter.

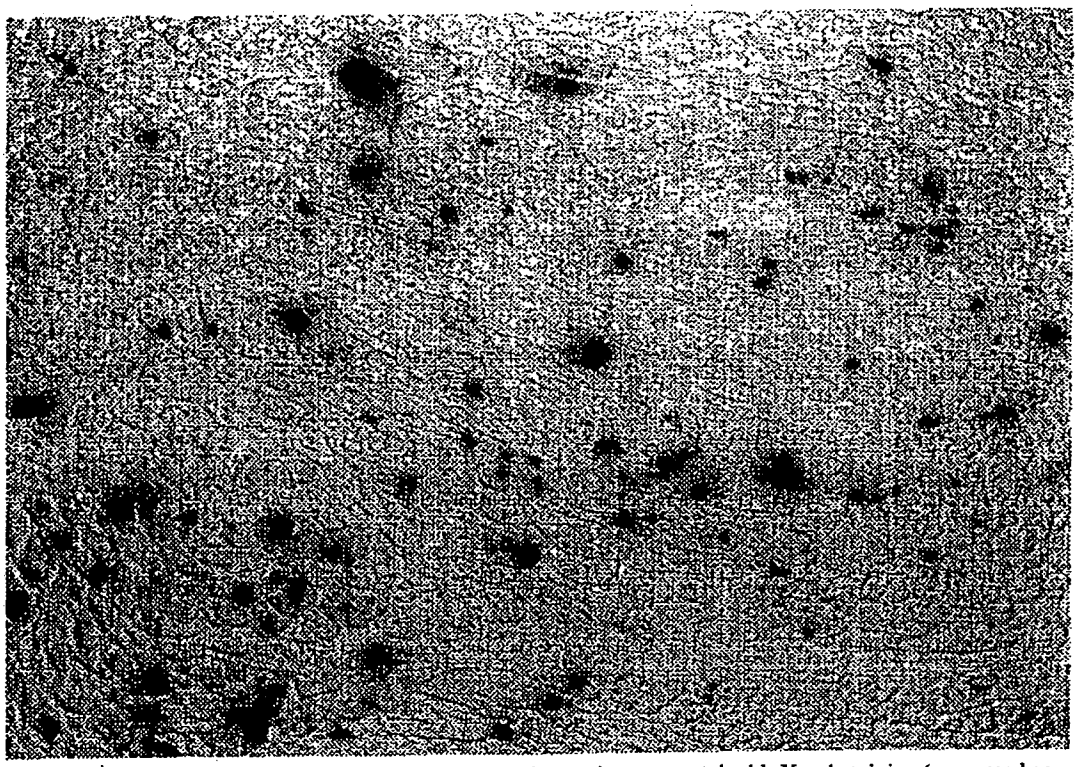


Fig. 1A. Successful gene transfer to the cardiac graft was demonstrated with X-gal staining (expressed as *blue spots*) in myocardial cells of coronary vessels.

Fig. 1B. Successful gene transfer to the cardiac graft was demonstrated with X-gal staining (expressed as *blue spots*) in endothelial cells of coronary vessels.

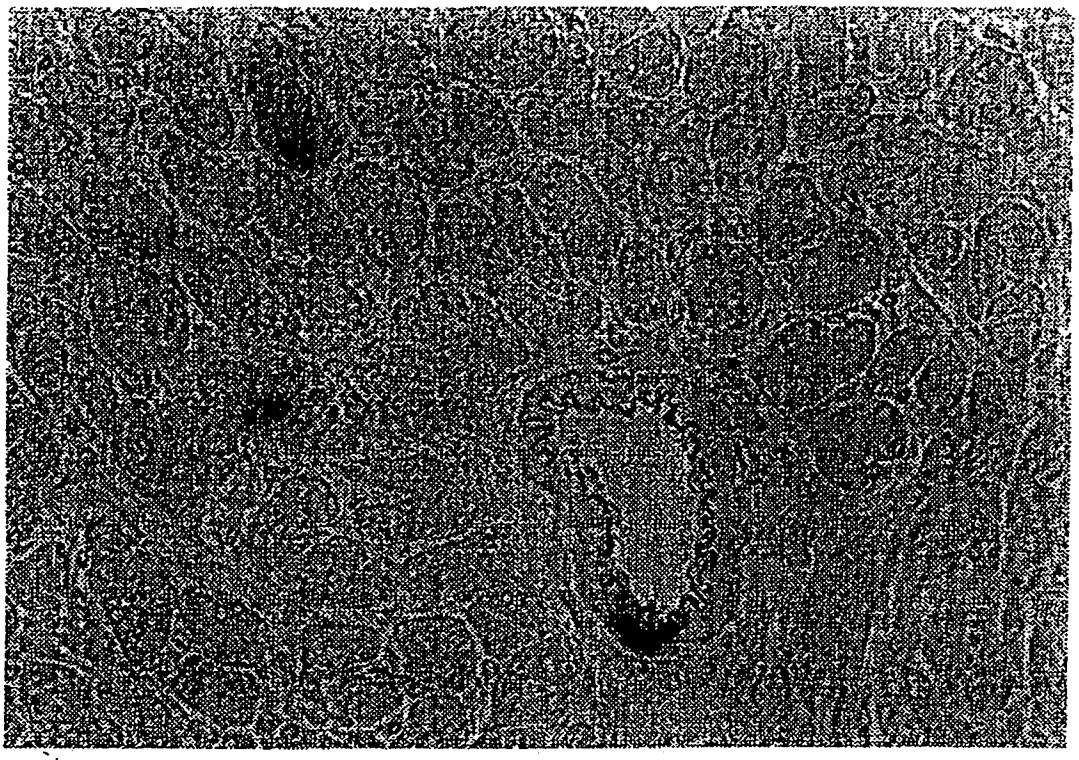


Fig. 1C. Successful gene transfer to the cardiac graft was demonstrated with X-gal staining (expressed as *blue spots*) in smooth muscle cells of coronary vessels.

time of the donor hearts ranged between 45 and 65 minutes. Nonsurvival rate, defined as survival for less than 24 hours after transplantation, was less than 10%. All animals surviving cardiac transplantation lived until they were sacrificed. All recipients had normal contractility of the transplanted heart when sacrificed. Aside from the β-galactosidase staining, the histologic appearances of both the experimental and control groups were normal.

Infection pattern and time course of expression. Successful gene transfer and expression of β-galactosidase was documented in all animals killed at 3 days. X-gal staining patterns revealed a nonuniform distribution, with blue-colored positively staining cells appearing in epicardial, myocardial, and endocardial cells, as well as in vascular walls (Figs. 1A, 1B, and 1C). The cells permissive to infection consisted predominantly of myocardial cells, and other myocardial structures were relatively well preserved (Fig. 1A). The mean total numbers of cells staining positively for β-galactoside per slice were 68.7 ± 27.3 in the 3-day group, 330.4 ± 53.8 in the 7-day group, 151.3 ± 48.0 in the 15-day group, and 39.9 ± 10.8 in the 30-day group, peaking in the 7-day group ($p < 0.05$). Control isografts ($n = 5$), retrieved at day 30, revealed no staining activity (Fig. 2).

Discussion

The present study proposes a model for adenovirus-mediated gene transfer to transplanted cardiac grafts and confirms the findings of recent studies on direct gene transfer into murine coronary arteries.[13] We observed expression of the *LacZ* gene, which is capable of producing the β-galactosidase enzyme, in the epicardial, myocardial, and endocardial cells, as well as in the coronary vascular walls, up to 30 days after transplantation, which demonstrates that this technique is a feasible means of transferring genes at the time of harvest.

Since Wolff and associates[15] demonstrated that murine skeletal muscle can take up and express genes that have been injected directly into the muscle, both skeletal and cardiac myocytes have been found to be efficient targets for gene transfer.[16] Several investigators have shown that expression of reporter genes injected into the skeletal or cardiac

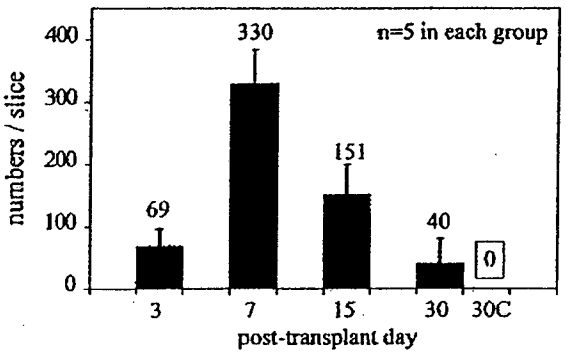


Fig. 2. Life span of transfected gene expression within the graft was analyzed by comparing the total number of cells staining positively for β-galactosidase per slice between the groups. Gene expression was detected in the 3-day group, peaking at 7 days, and gradually declined thereafter.

muscle was higher than for other injected tissues, including the brain, liver, spleen, uterus, stomach, lung, or kidney.[1, 2, 15] Although the reason for such apparently superior results remains unclear, it has been hypothesized that structural differences, including the presence of multinucleated cells, a sarcoplasmic reticulum, and a rich T-tubule system in skeletal and cardiac muscle, may allow better access of the gene to these cell populations.[1, 2, 15] The mechanism whereby the gene injectate is taken up by muscular tissues is unknown. It has been reported that only cells directly adjacent to the injection site are transfected, suggesting uptake of deoxyribonucleic acid (DNA) through leaking cell membranes.[1, 6] This finding, in addition to other experimental findings, raises questions about the clinical applicability of the direct injection method for transfection of human myocardium. These findings include the small number of myocytes that can be transfected in vivo (as few as 60 to 100 cells per injection), complete transfection only at the cells bordering the needle tract, and the occurrence of inflammation, myocardial necrosis, and scar formation along the needle tract.[1, 2]

Unlike direct myocardial injection, transfection via the walls of the coronary vasculature can be a useful means of gene transfer because of the potentially large absorptive area and widespread distribution. In vitro endothelial or vascular smooth muscle cells expressing a recombinant gene can be implanted on porcine endothelium-denuded vessel walls in vivo[8, 10] or on prosthetic interposition grafts of canine carotid arteries.[9] However, this technique requires prolonged occlusion of the target vessels to facilitate adenovirus attachment to the vascular wall,[8, 10] and it has a limited human application because of the practical difficulty of obtaining endothelial cells in advance.[11] Direct gene transfer into the unmodified vessels may overcome these problems. Previous reports have proved the feasibility of direct in vivo gene transfer into blood vessels.[11-13, 17] This study demonstrated that the recombinant adenovirus vector is able to transfer genes not only into the myocardial cells but also into the coronary arterial walls, the sites of local immune interactions after cardiac transplantation.

Recently, the adenovirus vector has been shown to be efficient in transferring exogenous genes into a variety of cells both in vitro and in vivo.[18-22] This vector has a number of advantages over other viral vectors. It is capable of infecting either nondividing or slowly proliferating cells, for example, vascular endothelial cells.[5, 23] Lemarchand and coworkers[10] have observed that replication-deficient adenovirus vector can mediate detectable gene expression in normal endothelial cells. The present study demonstrates that coronary vascular cells with β-galactosidase expression remain relatively intact microscopically. Other advantages of adenovirus vectors include the potential of generating viral stocks in excess of 1×10^{11} PFU/ml, an ability to accept heterologous genes up to 7.5 kb in length, no reported associations with human malignancies, and a history of safe administration in human vaccines.[24] Moreover, gene expression after adenovirus-mediated transfection in vivo could persist longer than other viruses. The mechanism underlying this phenomenon is unclear, but persistence of a moderate degree of viral replication may be responsible. A potential downside of this continued replication is that it may lead to a powerful host immune response to the infected cells.[23] A long-term model would be needed to examine this possible adverse effect.

An additional limitation regarding the generalized use of viral vectors has been the length of time required to generate each recombinant virus. Previous study has shown that an 18- to 24-hour incubation period is required to get the maximal lipofectin-mediated luciferase gene transfer in in vitro endothelial cell cultures.[11] Actually, such a long incubation period is not practical for in vivo gene transfer. In contrast, a considerable number (58%) of the endothelial cells expressed the β-galactosidase when the adenovirus vector (Ad.RSVβ-gal)

was in contact with the endothelial cells for even as short a period as 15 minutes in in vitro endothelial cell culture.[10] In the present study, the interval between the harvest and the reperfusion of the grafts (15 minutes of preservation in 4° C saline solution plus 45 minutes of room temperature exposure) was the incubation period. Clinically, the ischemic time of allografts provides a unique opportunity for access to the allografts and a window for direct gene transfer. Further studies are necessary, however, to demonstrate whether gene transfer can occur at hypothermic temperatures used for cardiac preservation.

The analysis of the time course of gene expression in our model corroborates the results obtained by others. Lemarchand and associates[10] observed that expression with adenovirus vectors peaked at 7 days and then declined, so that expression was no longer evident at 28 days in the in vivo carotid artery and umbilical vein of the lamb. Acsadi and coworkers[2] demonstrated that no β-galactosidase–positive cells were seen 25 days after direct myocardial injection of pRSVLacZ, suggesting that injected DNA remains episomal and is lost over time because of rapid target cell turnover. Additionally, they found that luciferase activity was stable for 60 days in cyclosporine-treated rats compared with unstable expression in untreated rats,[2] suggesting that an immune mechanism may be responsible for gene elimination. Nabel, Pautz, and Nabel[25] observed that β-galactosidase activity was expressed for at least 5 months after they transfected a segment of the iliofemoral arteries with retrovirus in vivo, and all three layers of the vessels including endothelial and vascular smooth muscle cells were infected by the retrovirus. Further efforts to increase the functional life span of viruses injected into infected organs need to be explored.

In summary, our model demonstrates that intracoronary gene transfer to the transplanted murine cardiac grafts is feasible at harvest. Adenovirus-mediated gene transfer produces widespread gene expression which, though perhaps transient, does not adversely affect myocardial structure or function. This technology may allow modification of graft immunogenicity in the future by using gene sequences capable of encoding immunosuppressive proteins. However, further efforts directed at increasing the level of expression and the functional life span of gene sequences need to be applied for this technique to be useful in patients.

REFERENCES

1. Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 1990; 98:2217-21.
2. Acsadi G, Jiao S, Jani A, et al. Direct gene transfer and expression into rat heart in vivo. New Biol 1991;3:71-81.
3. Wang J, Jiao S, Wolff JA, Knechtle SJ. Gene transfer and expression in rat cardiac transplants. Transplantation 1992;53:703-5.
4. Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA. Behavior of genes directly injected into rat heart in vivo. Circ Res 1992;70:193-8.
5. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus. Circ Res 1993;73: 1202-7.
6. Gal D, Weir L, Leclerc G, Pickering JG, Hogan J, Isner JM. Direct myocardial transfection in two animal models: evaluation of parameters affecting gene expression and percutaneous gene therapy. Lab Invest 1993;18:18-25.
7. von Harsdorf R, Schott RJ, Shen Y-T, Vatner SF, Mahdavi V, Nadal-Ginard B. Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals. Circ Res 1993;72:688-95.
8. Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ. Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 1989;244: 1342-4.
9. Wilson JM, Birinyi LK, Salomon RN, Libby P, Callow AD, Mulligan RC. Implantation of vascular grafts lined with genetically modified endothelial cells. Science 1989;244:1344-6.
10. Lemarchand P, Jones M, Yamada I, Crystal RG. In vivo gene transfer and repression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. Circ Res 1993;72:1132-8.
11. Lim CS, Chapman GD, Gammon RS, et al. Direct in vivo gene transfer into coronary and peripheral vasculatures of the intact dog. Circulation 1991;83:2007-11.
12. Chapman GD, Lim CS, Gammon RS, et al. Gene transfer into coronary arteries of intact animals with a percutaneous balloon catheter. Circ Res 1992;71:27-33.
13. Ardehali A, Fyfe AI, Laks H, Drinkwater DC, Qiao JH, Lusis AJ. Direct gene transfer into donor hearts at the time of harvest. J Thorac Cardiovasc Surg 1995;109:716-9.
14. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 1990;10:4239-42.
15. Wolff JA, Malone RW, Williams P, et al. Direct gene

transfer into mouse muscle in vivo. Science 1990;247: 1465-8.
16. Stratford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart. J Clin Invest 1992; 90:626-30.
17. Flugelman MY, Jaklitsch MT, Newman KD, Casscells W, Bratthauer GL, Dichk DA. Low level in vivo gene transfer into arterial wall through a perforated balloon catheter. Circulation 1992;85:1110-7.
18. Kirshenbaum LA, MacLellan WB, Majur W, French BA, Schneider MD. Highly efficient gene transfer into adult ventricular myocytes by recombinant adenovirus. J Clin Invest 1993;92:381-7.
19. La Salle GLG, Robert JJ, Berrad S, et al. An adenovius vector for gene transfer into neurons and glia in the brain. Science 1993;259:988-90.
20. Rosenfeld MA, Siegfried W, Yoshimura K, et al. Adenovirus-mediated transfer of a recombinant α_1-antitrypsin gene to the lung epithelium. Science 1991; 252:431-4.
21. Smythe WR, Kaiser LR, Hwang HC, et al. Successful adenovirus mediated gene transfer in an in vivo model of human malignant mesothelioma. Ann Thorac Surg 1994;57:1395-401.
22. Haddada H, Lopez M, Martinache C, Ragot T, Abina MA, Perricaudet M. Efficient adenovirus mediated gene transfer into human blood monocyte-derived macrophage. Biochem Biophys Res Commun 1993; 195:1174-83.
23. Mulligan RC. The basic science of gene therapy. Science 1993;260:926-32.
24. Berkner KL. Development of adenovirus vectors for expression of heterogenous genes. Biotechniques 1988;6:616-29.
25. Nabel EG, Pautz G, Nabel GY. Gene transfer into vascular cells. J Am Coll Cardiol 1991;17:189B-94B.

Discussion

Dr. John E. Mayer *(Boston, Mass.)*. Genetic modification of donor allografts by means of viral vectors offers an exciting potential avenue by which both acute and chronic rejection may be modified.

Two questions have occurred to me. First, although much of your interest seems to have been focused on endothelial cells and transplant atherosclerosis, the great majority of infected cells were myocytes. Do you have any explanation for this? Does this finding imply that a different viral vector may be necessary or should the dose of the virus be greater?

Second, can you speculate on which genes should be transferred into the donor hearts? I have wondered whether the donor cells could be induced to produce the same HLA antigens as the recipient. Do you have any other ideas concerning which genes could be transferred into the donor organ?

Dr. Lee. Dr. Mayer, thank you for your comments and questions. First, our model was the first step study to evaluate the spacial and temporal expression pattern of adenovirus-mediated gene transfer in the transplanted mouse heart. It is true that the endothelial cells are thought to be among the useful targets to transfect genes to modulate local immune responses after cardiac transplantation because of their large absorptive area and widespread distribution. Recently, adenovirus vector has been shown to be an efficient tool to transfect genes even into nondividing or slowly dividing cells like myocardial or endothelial cells in both in vivo and in vitro studies. As you commented, our results revealed that myocardial cells were more efficiently transfected than the endothelial cells. However, the gene expressions in both myocardium and endothelium of the coronary vascular beds, the sites of local immune responses, were demonstrated in this study. This may justify the possible role of this approach to treat either transplantation coronary artery disease or graft rejection. The relative efficiency of transfection to the different cell types is to be studied further.

Second, there is no doubt that the long-term goal of these experiments is to develop the methods to express the transferred genes and to produce biologically active proteins in quantities sufficient to modulate local immune responses. Application of this technology using gene sequences for immunosuppressive cytokines including transforming growth factor-β (TGF-β) and interleukin-10 is currently underway with favorable results. Further efforts should be directed toward ways to increase the functional life span and rate of infection of these inserted gene sequences for this technology to be clinically applicable.

Dr. John H. Kennedy *(Cambridge, England)*. Homocystine has recently been implicated as a risk factor in atherosclerosis, and the cytomegalovirus family of viruses is thought to invoke this response. Homocystine remains in tissue or body fluids for as long as 10 years. If you have any frozen material, it might be interesting to see whether a different virus would be more useful in your particular model if you are interested in graft rejection and accelerated atherosclerosis.

EXHIBIT Q

Gene Therapy (1996) 3, 560–566

Gene delivery to the heart in vivo and to cardiac myocytes and vascular smooth muscle cells in vitro using herpes virus vectors

RS Coffin[1], MK Howard[1], DVE Cumming[1,2], CM Dollery[2], J McEwan[2], DM Yellon[2], MS Marber[3], AR MacLean[4], SM Brown[4] and DS Latchman[1]

[1]*Department of Molecular Pathology, University College London Medical School, The Windeyer Building, 45 Cleveland Street, London W1A 6DB;* [2]*The Hatter Institute for Cardiovascular Studies, London;* [3]*Department of Cardiology, Guy's and St Thomas' Hospital Medical Schools, London; and* [4]*The MRC Institute of Virology, Glasgow, UK*

Herpes simplex virus 1 (HSV1), while usually thought of as neurotropic, can also efficiently infect a wide variety of non-neuronal cell types and so might be developed as a vector for gene delivery to non-neuronal as well as neuronal cells. Here we have tested three different disabled HSV vectors for their ability to deliver a lacZ gene to primary cardiac myocytes and vascular smooth muscle cells in vitro and used the most efficient virus to transfect the rat heart in vivo. We also assessed the degree of cytopathic effect of the various viruses on the cardiac myocytes in vitro by testing the effects on the frequency of beating in synchronously beating myocyte cultures. While an HSV mutant in which the essential immediate-early gene IE2 had been deleted gave high efficiency gene transfer to the cardiac myocytes in vitro and the rat heart in vivo, viruses in which ICP34.5 or ICP34.5 and VMW65 were inactivated (and which were also unable to replicate in these cells) gave a much lower efficiency of gene transfer, mirroring the degree of cytopathic effect observed in the beating myocyte cultures. Gene transfer to the vascular smooth muscle cells was considerably less efficient than to the myocytes in all cases. These results indicate that while HSV may be inappropriate for highly efficient gene transfer to the arterial wall, efficient gene transfer can be achieved in the myocardium, and thus that HSV vectors may be suitable for the alteration of cardiac cell physiology in vivo.

Keywords: *herpes virus vectors; cardiac myocytes; vascular smooth muscle cells; gene therapy*

Introduction

A variety of methods are under development for gene transfer to the myocardium and coronary vasculature, including the use of adenoviral vectors and the direct injection of plasmid DNA (reviewed by Nabel[1]), where it is hoped that they may in the future allow the treatment of a number of cardiovascular diseases through the expression of therapeutic gene products. Example conditions for which gene therapeutic protocols can be envisaged include ischaemic heart disease or in restenosis prevention following percutaneous transluminal coronary angioplasty.

Herpes simplex virus 1 (HSV1) has been proposed as a candidate vector for gene delivery to the nervous systems (reviewed by Coffin and Latchman[2]) as it can produce a lifelong latent infection in neurons. However, HSV1 can also infect a wide range of other cell types and could therefore be used for gene delivery to non-neuronal cells as long as lytic infection were inhibited. Indeed HSV has recently been used to deliver *lacZ* to mouse smooth muscle cells *in vitro* and *in vivo*.[3] Moreover the particularly stable DNA structure formed by nonreplicating HSV genomes may be particularly suitable for allowing long-term genetic correction in nondividing cells. HSV is highly pathogenic and thus must, like most other viral vectors, be disabled in some way. Most replication defective HSV-1 vectors contain a deletion to remove one or more immediate-early (IE) genes in order to prevent virus replication at the earliest possible time after infection. These genes must be complemented *in trans* for growth in culture. Alternatively, either an inactivating mutation in the gene encoding VMW65 (which transactivates IE genes after infection) can be used to produce a nonpathogenic virus, or genes necessary for replication in particular target cell types can be removed. A mutation of this last type is provided by removal of the ICP34.5 gene which prevents replication in some fully differentiated cell types (including neurons) but which still allows virus growth in actively dividing fibroblasts in culture.[4] Here we have tested disabled viruses of these (or combinations of the three) types for the ability to direct β-galactosidase (*lacZ*) expression in cultured rat cardiac myocytes, primary rat aortic vascular smooth muscle cells (VSMCs), and a VSMC cell-line *in vitro*, and also tested the most efficient virus in the rat heart *in vivo*. These viruses were either deleted for ICP34.5, ICP34.5 with VMW65 inactivated, or deleted for IE2 (encoding essential immediate-early protein ICP27). Thus whil

Correspondence: RS Coffin
Received 11 January 1996; revised 26 February 1996; accepted 1 March 1996

wild-type HSV is highly neurotrophic, these various deletions of the HSV genome would prevent any possible neurological complications when using such vectors for gene delivery to non-neuronal cells. However, while ICP34.5-deleted viruses have already been tested and been shown to give safe transgene delivery to the mouse and rat central and peripheral nervous systems *in vivo* (manuscript submitted), their efficiency for gene delivery, their replication status and the degree of cytopathic effect (CPE) produced in cardiac cells or VSMCs were unknown at the begining of this study.

Results

Experiments were performed both to assess the efficiency of gene transfer to the cardiomyocytes and vascular cells and to assess the degree of cytotoxicity of the various viruses. Thus cell cultures were infected with a number of viruses carrying the *lacZ* gene: a nondisabled virus (BE8),[5] viruses disabled by the removal of ICP34.5[6] or ICP34.5 together with the inactivation of VMW65[7] (1716/*lacZ* or 1764/*lacZ*), or a virus with the essential IE gene encoding ICP27 inactivated (27-*lacZ*).[8] Cells were then either stained with X-gal at 1 or 3 days and blue cells counted as a means of assessing the efficiency of gene delivery, or the health of the culture roughly assessed by counting the frequency of beating (beats per min) in synchronously contracting myocyte cultures, as compared with an uninfected control at 1 day, 2 days and 5 days after infection. Healthy, recently isolated cultures of rat newborn cardiomyocytes contract in a regular and synchronous fashion for a number of days, although they are highly sensitive to physiological or other insult during this time. The degree of virus replication was assessed by removal of the supernatant from the cultures and titration (followed by plaque counting) on a susceptible cell line (BHK C-13, or BHK C-13 expressing ICP27).

X-gal staining

Bearing in mind the beat frequency data below (see Table 1), which shows that at multiplicities of infection (MOI) >1 there is considerable cytotoxicity even for the disabled viruses, the X-gal staining experiments were all performed at MOI = 1. As can be seen from Figure 1a, all the viruses can deliver *lacZ* to the cardiomyocytes, although in accordance with the beat frequency data below, the efficiency of gene transfer varies significantly. However as in each case (except for BE8) there was little difference between the numbers of blue cells after 1 day or 3 days, it appeared likely that all the disabled viruses were incapable of replication in the cardiomyocytes. This was confirmed by the virus titration experiments which showed that while large numbers of plaques were generated on BHK cells by the supernatant harvested from cardiomyocytes 3 days after inoculation with BE8, no plaques were generated from the supernatant after inoculation with 1716/*lacZ*, 1764/*lacZ* or 27-*lacZ* (in this last case titrated on a BHK cell line expressing ICP27).

The nondisabled virus (BE8) gave a high proportion of stained cells, but with a high degree of accompanying cell death, showing not only the ability of HSV to infect and replicate in cardiomyocytes, but also the requirement for effective disablement of an HSV vector. However, while the viruses with either ICP34.5 or ICP34.5 and VMW65 removed gave only a relatively low number of blue staining cells, 27-*lacZ* produced both a very high percentage of blue cells and little visible CPE. This suggests that the absolute block to replication provided by this mutant, and the probable lower level of virus gene expression is more effective at generating an efficient and non-cytopathic vector for cardiac cells than removal of ICP34.5 or ICP34.5 and VMW65. This difference may be due to the specific function of ICP34.5 in some cell types (see Beat frequencies and Discussion) which might prevent efficient *lacZ* expression.

While the primary rat cardiomyocytes were evidently highly infectable by HSV (either wild-type or disabled), the primary aortic vascular smooth muscle cells were considerably less susceptible, as even the nondisabled virus gave only a considerably lower percentage of X-gal staining cells even at higher multiplicities of infection than used for the cardiocytes above (MOI of 5 instead of 1; Figure 1b). However, of the disabled viruses 27-*lacZ* again gave the best results, with only a few cells staining with X-gal when infected with the ICP34.5 or ICP34.5/VMW65 deleted virus (again at MOI = 5). While these primary cultures of vascular smooth muscle cells could be infected by HSV, albeit at lower efficiency than the cardiomyocytes, the vascular smooth muscle cell line used gave no blue cells even at MOI > 10 with 27-*lacZ*, and only a few blue cells when infected with BE8 at high MOI. HSV is therefore inappropriate for gene delivery to these cells in culture.

Beat frequencies

Table 1 shows the results of the beat frequency experiments after inoculation of the cardiomyocytes with the various viruses at multiplicities of infection (MOI) of 0.5, 1 and 5. The nondisabled virus (BE8) showed marked cytotoxicity after 1 day, with little and irregular beating at MOI = 1 and no beating at MOI = 5. By day 2, some recovery had occurred at MOI = 1 although beating was slightly higher than mock-infected frequencies (approximately 200, as against approximately 150) and still irregular. All the cells were dead by day 5. 1716/*lacZ* and 1764/*lacZ* showed beat frequencies at near to mock-infected levels at MOI ≤ 1 after 1 day, although at higher multiplicities of infection beat frequency was reduced. At day 2, the cells infected at lower multiplicity were still beating although at below mock levels, while with the higher multiplicities beating had essentially stopped. The cells were again dead by day 5. The removal of ICP27 gave a considerable improvement in cell survival and beat frequency at MOI ≤ 1, with at day 1 near to mock levels, day 2 half way between mock and 1716/*lacZ* or 1764/*lacZ* levels, and at day 5 the cells were still beating slowly. At higher MOI, beat frequencies were more markedly reduced at all time-points. However, at day 5 even mock-infected cultures are no longer beating stably, with high speed, unsynchronised contractions (fibrillation).

These results show that while all the disabled viruses are incapable of replication in the cardiomyocytes (see above), only the removal of ICP27 generates a vector in which the toxic effects of infection have been reduced significantly, as at an MOI ≤ 1 beat frequencies are similar to mock-infected controls. The deletion of ICP34.5 or ICP34.5 and VMW65, while preventing replication, does not provide a vector in which the cytotoxic effects of infection have been very significantly reduced (although the effects are considerably lower than with BE8 which

(a)

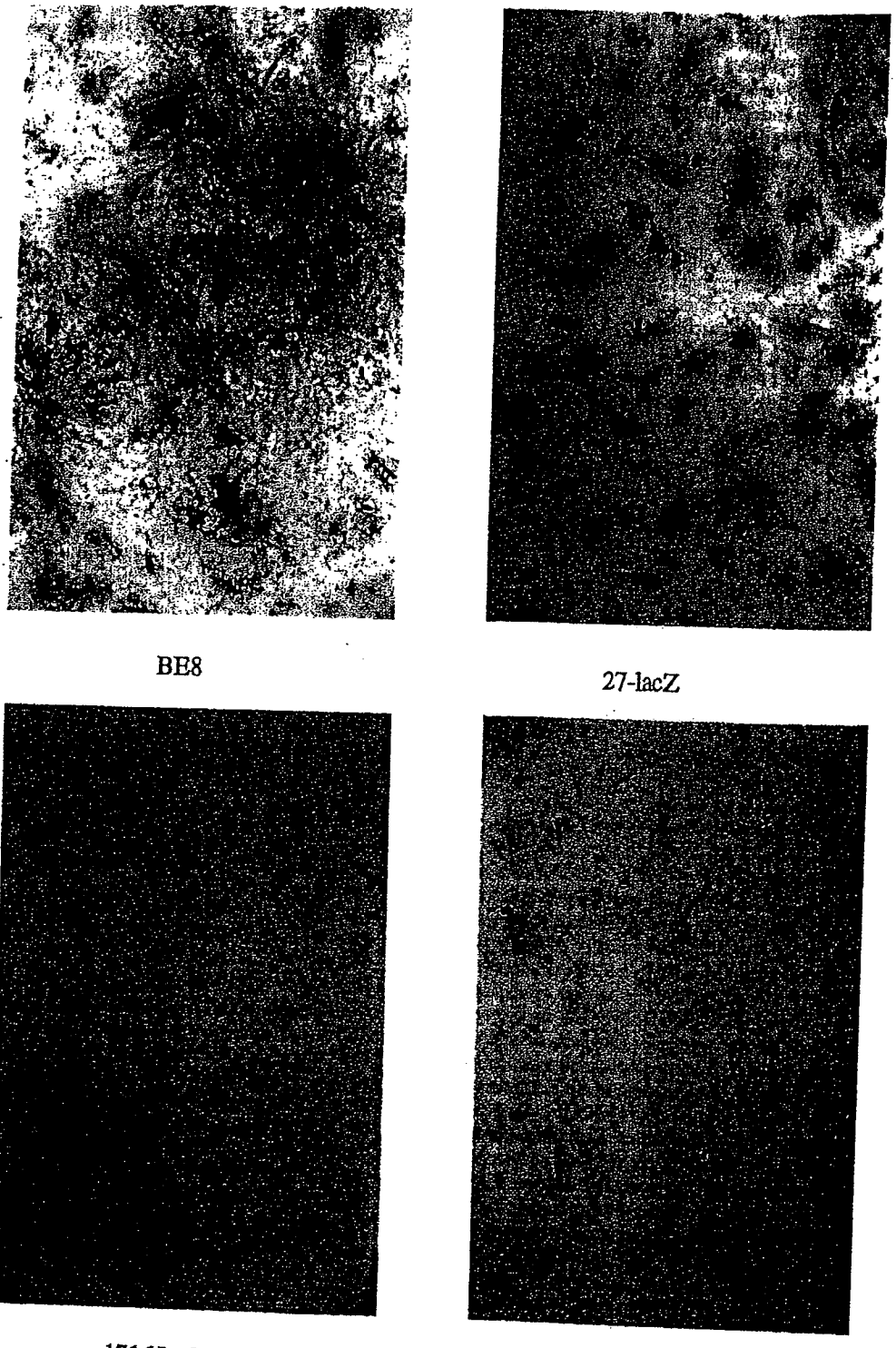
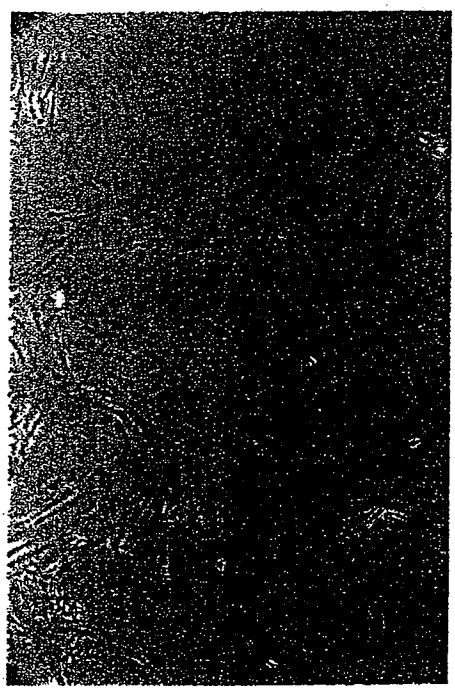


Figure 1 (a) Newborn rat primary cardiac myocyte cultures or (b) primary vascular smooth muscle cultures stained with X-gal 3 days after infection with either BE8, 27-lacZ, 1716/lacZ or 1764/lacZ. The primary cardiac myocytes were infected at MOI = 1 and the vascular smooth muscle cells at MOI = 5.

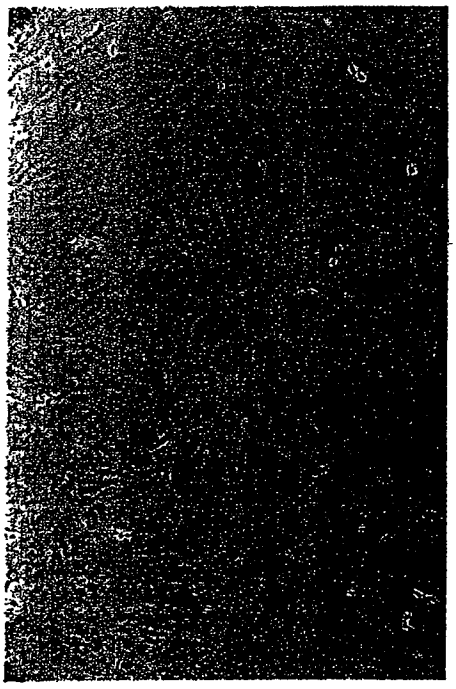
(b)

BE8

27-lacZ



1716/lacZ



1764/lacZ

Figure 1 (Continued).

Table 1 Frequency of synchronous beating in newborn rat primary cardiac myocyte cultures 1 day, 2 days or 5 days after infection with either DB8, 1716/*lacZ*, 1764/*lacZ* or 27-*lacZ* at various MOIs

MOI	DB8		1716/*lacZ*			1764/*lacZ*			27-*lacZ*			Mock
	1	5	0.5	1	5	0.5	1	5	0.5	1	5	
Day 1	5 irregular	0	162	145	43	164	158	62	160	163	100	160
Day 2	204 irregular	0	48	40	7 irregular	52	47	5 irregular	150	135	58	153
Day 5	0	0	0	0	0	0	0	0	100	50	0	276 fibrillating

gives a lytic infection in these cells). This might be for two reasons: firstly removal of ICP34.5 (and VMW65 in the case of 1764/*lacZ*) may still allow the expression of many potentially cytotoxic HSV gene products, particularly the IE genes, which may account for the effects we have observed, or secondly in the absence of ICP34.5 protein synthesis might be shutdown in the infected cells which might also account for the effects on beat frequency. This type of response has been noted previously in a neuronally derived cell line[9] (although it is not seen in other nonpermissive cells),[10,11] where it was suggested that the natural response to infection by an ICP34.5-deleted virus in these cells was a shutdown in protein synthesis, possibly leading to apoptosis, which would limit the extent of the infection in the host, but which was blocked by the expression of ICP34.5.

In vivo gene delivery to the heart

To assess whether the apparent utility of HSV vectors for the heart *in vitro* was reflected in efficient delivery to the heart *in vivo*, the most successful mutant *in vitro* (deleted for ICP27) was used *in vivo* in the rat heart. For these experiments intramyocardial injections were made directly through the chest wall of anaesthetised adult Lewis rats after locating the heart by palpation. The rats were allowed to recover and after 2 days were killed, the heart removed, fixed and stained with X-gal. As can be seen in Figure 2 efficient gene delivery can be achieved in the adult rat myocardium as large areas staining with X-gal can be seen around the inoculated site. The rats remained healthy during the 2-day incubation period and the X-gal stained areas remained healthy in appearance with no apparent alteration in cell morphology suggesting any cytopathic effects of the inoculated virus to be minimal. However, as would be expected from a nonreplicating viral vector, the area of staining is limited to the immediate vicinity of the inoculated site, suggesting that multiple injections or other means of delivery may be required for significant physiological effects if such a vector were to be used for the delivery of, for example, genes for the 70 kDa heat shock protein (HSP70) which might protect against the effects of ischaemia.[12,13]

Figure 2 In vivo gene delivery to the rat myocardium. Rat hearts were inoculated as described in the text and fixed and stained with X-gal 2 days after inoculation with 27-lacZ.

Discussion

HSV has traditionally been regarded as an ideal candidate vector for the nervous system, as with suitable development its neurotrophic life-style and its ability to

maintain a lifelong latent infection might provide a means by which neuronal physiology could be altered in the long term after a once-only application of such a vector system. However HSV can also efficiently infect many other cell types and in some circumstances might provide specific advantages over other vector systems, particularly in the delivery of genes to terminally differentiated cells such as those of the myocardium, where very long-term gene expression from latent virus might be envisaged, or where large genetic insertions might be required. HSV does not have the packaging constraints of other viruses, potentially allowing the use of large control DNA regions which might be important for correct cell type-specific gene expression. Indeed HSV has recently been shown to be efficient at delivering *lacZ* to skeletal muscle cells *in vivo*.[3] However, to exploit HSV as a vector it must be disabled to minimise the effects on the target cell, and a number of strategies for this have previously been employed. These have included the deletion of essential genes, allowing virus growth only in a complementing cell line and the deletion of genes which specifically prevent growth in target cells but still allow growth in culture. For neurons these have included genes such as ICP6,[14] thymidine kinase[15] and ICP34.5,[4] only the last of which provides an absolute block to a productive infection in these cells. Here we have tested and compared viruses disabled by both these means and showed that while viruses deleted for the essential IE gene ICP27 (and therefore grown on ICP27 expressing cells) provide efficient and relatively noncytopathic vectors for cardiomyocytes *in vitro* and the rat myocardium *in vivo*, deletion of ICP34.5, while preventing replication in these cells, does not allow efficient gene delivery. We have also shown that *in vitro* HSV is less efficient for gene transfer to VSMCs, although this was not tested *in vivo*.

It has been speculated that delivery of a number of genes to the myocardium, particularly HSPs, might protect against the effects of ischaemia,[12] and indeed transgenic mice overexpressing HSP70 are significantly protected.[13] It has also been shown that fibroblast growth factor (FGF) when delivered to porcine iliofemoral arteries can induce angiogenesis.[16] While a number of vector systems have been tested for *in vitro* or *in vivo* gene delivery, including adenoviral vectors, retroviral vectors, liposomes or the direct injection of plasmid DNA (reviewed by Nabel[1]), none provides an optimal gene delivery system under all circumstances. HSV has previously been shown to allow delivery of *lacZ* in a nonvascularised cardiac transplantation model in the mouse where neonatal whole hearts were removed, inoculated with virus, and placed subcutaneously in the ear pinna of recipient mice.[17] Our experiments further show that HSV could be developed as a vector system to deliver some or all of these genes to the myocardium, and that it might provide advantages in some circumstances, for example for long-term or tissue-specific expression. For these reasons we are currently developing herpes vectors to express a number of HSPs and FGF to allow the potential of HSV for altering cardiac cell physiology to be tested *in vivo*.

Materials and methods

Cardiocyte cell culture

Ventricular myocytes from the hearts of <2-day-old neonatal Sprague–Dawley rats were isolated and cultured as previously described.[18–20] Cardiocyte cultures under these conditions start to beat in synchrony within 24–48 h of isolation, the percentage of beating cells exceeding 85% for the duration of the experiment. Cells grown on laminin-coated coverslips were used for immunofluorescent staining with an antimyosin antibody (Amersham International, Amersham, UK) to confirm the proportion of cardiocytes staining for myosin heavy chain.

Vascular smooth muscle cell (VSMC) cultures

Primary VSMCs were produced by removal of the thoracoabdominal aortas of 3-month-old male Wistar rats which were then stripped of endothelium and adventitia. Medial VSMCs were obtained by the combined collagenase/elastase digestion method.[21] Isolated cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, 50 mg/ml penicillin, 50 mg/ml streptomycin (all Gibco, Gaithersburg, MD, USA) at 37°C with 5% CO_2. VSMCs were identified by their characteristic morphology and immunostaining for sm-myosin (M-7648 from Sigma, Poole, UK). At confluence, cells were passaged using 0.25% trypsin, 3 mM EDTA and were used at passage 4–6 for these experiments. The VSMC cell line used is an SV40-transformed rat abdominal aorta smooth muscle culture obtained from the American Type Culture Collection (ATCC-CRL2018, Rockville, MD, USA).

Virus stocks

Herpes virus strains used were BE8 (an essentially wild-type virus with a CMV IE promoter/*lacZ* insertion into a nonessential gene (US5))[5] and three disabled viruses 1716/*lacZ*, 1764/*lacZ* and 27-*lacZ*.[8] 1716/*lacZ* and 1764/*lacZ* were produced by the insertion of a chimaeric herpes latently active transcript (LAT)-Moloney murine leukaemia virus long terminal repeat (MoMLVLTR) promoter/*lacZ* cassette into the UL43 gene of HSV1 strains 1716 and 1764 respectively, by standard methods.[2] 1716 Is deleted for both copies of ICP34.5,[6] and 1764 also has an inactivating insertional mutation in the gene for VMW65.[7] UL43 is a nonessential HSV gene, unnecessary for growth in culture and which does not affect the kinetics of the establishment or reactivation from latency *in vivo*.[22] 27-*lacZ* has an ICP6 promoter/*lacZ* insertion into the gene encoding the essentail IE protein ICP27.[8]

In vivo inoculation

Approximately 20 µl intramyocardial injections of 27-*lacZ* (5×10^8 p.f.u./ml) were made directly through the chest wall of anaesthetised adult Lewis rats after accurately locating the heart by palpation. The rats were allowed to recover and after 2 days were killed, the heart removed, fixed and stained with X-gal.

X-gal staining

Culture medium was removed from the cells which were then fixed in 4% paraformaldehyde at room temperature for 15 min. After washing twice in phosphate buffered saline (PBS), cells were then stained with X-gal for approximately 1 h at 37°C in 5 mM potassium ferricyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride, 0.02% NP40, 0.02% sodium deoxycholate, 1 mg/ml X-gal (Sigma, Poole, UK) in PBS. Whole hearts inoculated *in vivo* were removed from the animal after death

and treated similarly except that the fixing step was extended to 1 h and incubation in X-gal to 4 h.

Acknowledgements

RSC is supported by the Parkinsons Disease Society, DVEC and MKH by the Wellcome Trust, MSM by the British Heart Foundation and CMD by the Medical Research Council.

References

1 Nabel EB. Gene therapy for cardiovascular diseases. *Circulation* 1995; 91: 541–548.
2 Coffin RS, Latchman DS. Herpes simplex virus-based vectors. In: Latchman DS (ed). *Genetic Manipulation of the Nervous System*. Academic Press: London, 1996, pp 99–111.
3 Huard J, Goins WF, Glorioso JC. Herpes simplex virus type 1 vector mediated gene transfer to muscle. *Gene Therapy* 1995; 2: 385–392.
4 Chou J, Kern ER, Whitley RJ, Roizman B. Mapping of herpes simplex virus-1 neurovirulence to γ34.5, a gene nonessential for growth in culture. *Science* 1990; 250: 1262–1266.
5 Balan P *et al*. An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *J Gen Virol* 1994; 75: 1245–1258.
6 MacLean AR *et al*. Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence related sequences in Glasgow strain 17+ between immediate–early gene 1 and the 'a' sequence. *J Gen Virol* 1991; 72: 632–639.
7 Ace CI *et al*. Construction and characterisation of a herpes simplex virus type 1 mutant unable to transinduce immediate–early gene expression. *J Virol* 1989; 63: 2260–2269.
8 Smith IL, Hardwicke MA, Sandri-Goldin RM. Evidence that the herpes simplex virus immediate–early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. *Virology* 1992; 186: 74–86.
9 Chou J, Roizman B. The γ34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc Natl Acad Sci USA* 1992; 89: 3266–3270.
10 Brown SM *et al*. Cell type and cell state determine differential *in vitro* growth of non-neurovirulent ICP34.5-negative herp simplex virus types 1 and 2. *J Gen Virol* 1994; 75: 2367–2377.
11 Brown SM, MacLean AR, Aitken JD, Harland J. ICP34.5 inf ences herpes simplex virus type 1 maturation and egress fr infected cells *in vitro*. *J Gen Virol* 1994; 75: 3679–3686.
12 Marber MS, Walker JM, Latchman DS, Yellon DM. Myocard protection after whole body heat stress in the rabbit is dep dent on metabolic substrate and is related to the amount of i inducible 70-kD heat stress protein. *J Clin Invest* 1994; 1087–1094.
13 Marber MS *et al*. Overexpression of the rat inducible 70 kilod ton heat stress protein in a transgenic mouse increases the resi ance of the heart to ischaemic injury. *J Clin Invest* 1995; 1446–1456.
14 Goldstein D, Weller SK. Herpes simplex virus type 1-induc ribonucleotide reductase activity is dispensable for virus grow and DNA synthesis: isolation and characterisation of an IC *lacZ* insertion mutant. *J Virol* 1988; 62: 196–205.
15 Coen DM *et al*. Thymidine kinase-negative herpes simplex vir mutants establish latency in mouse trigeminal ganglia but not reactivate. *Proc Natl Acad Sci USA* 1989; 86: 4736–4740.
16 Nabel EG *et al*. Recombinant FGF-1 promotes intimal hyp plasia and angiogenesis in arteries *in vivo*. *Nature* 1993; 3 844–846.
17 Qin L *et al*. Multiple vectors effectively achieve gene transfer a murine cardiac transplantation model. *Transplantation* 1995; 809–816.
18 Chien KR *et al*. Release of arachidonate from membrane ph pholipids in cultured neonatal rat myocardial cells during ad osine triphosphate depletion. *J Clin Invest* 1985; 75: 1770–17
19 Buja LM *et al*. Alterations of ultrastructure and elemental co position in cultured neonatal rat cardiac myocytes after me bolic inhibition with iodoacetic acid. *Lab Invest* 1988; 53: 397–4
20 Knowlton KU *et al*. Co-regulation of the atrial natriuretic fac and cardiac myosin light chain-2 genes during a adrener stimulation of neonatal rat ventricular cells. *J Biol Chem* 19 266: 7759–7768.
21 Rennick RE, Cambell JW, Cambell GR. Vascular smooth mus phenotype and growth behaviour can be influenced by mac phages *in vitro*. *Atherosclerotion* 1988; 71: 35–43.
22 MacLean CAS *et al*. Investigation of herpes simplex virus ty 1 genes encoding multiply inserted membrane proteins. *J G Virol* 1991; 72: 897–906.

EXHIBIT R

CARDIAC AND PULMONARY REPLACEMENT

EX VIVO ADENOVIRUS-MEDIATED GENE TRANSFER TO THE ADULT RAT HEART

Alan P. Kypson, MD[a]
Karsten Peppel, PhD[b]
Shahab A. Akhter, MD[a]
R. Eric Lilly, MD[a]
Donald D. Glower, MD[a]
Robert J. Lefkowitz, MD[b,c]
Walter J. Koch, PhD[a]

Sponsor:
Robert W. Anderson, MD[a]

Objective: The ability to transfer genes to adult myocardium may have therapeutic implications for cardiac transplantation. We investigated the feasibility of adenovirus-mediated transfer of marker genes *LacZ* and *Luciferase*, as well as the potentially therapeutic gene of the human β_2-adrenergic receptor in a rat heterotopic heart transplant model. *Methods:* Donor hearts were flushed with 10^{12} total viral particles of one of three transgenes. Hearts were harvested at various time points after transplantation. *LacZ*-treated hearts were assessed by histologic staining and *Luciferase*-treated hearts were assayed for specific luminescence activity. Hearts treated with β_2-adrenergic receptor underwent radioligand binding assays and immunohistochemistry with the use of an antibody specific for the human β_2-adrenergic receptor. *Results: LacZ* hearts revealed diffuse myocyte staining as opposed to none within controls at 5 days. *Luciferase* hearts demonstrated a mean activity of 970,000 ± 220,000 arbitrary light units versus 500 ± 200 for the controls ($p = 0.001$). Total β_2-adrenergic receptor densities (fmol/mg membrane protein) for hearts that received the β_2-adrenergic receptor transgene at 3, 5, 7, 10, and 14 days after infection were as follows: right ventricle, 488.5 ± 126.8, 519.4 ± 81.8,* 477.1 ± 51.8,* 183.0 ± 6.5,* and 82.7 ± 19.1; left ventricle, 511.0 ± 167.6, 1206.4 ± 321.8,* 525.3 ± 188.7, 183.5 ± 18.6,* and 75.9 ± 15.2 (*$p < 0.05$ vs control value of 75.6 ± 6.4). Immunohistochemical analysis revealed diffuse staining of varying intensity within myocardial sarcolemmal membranes. *Conclusions:* We conclude that global overexpression of different transgenes is possible during cardiac transplantation and, ultimately, adenovirus-mediated gene transfer may provide a unique opportunity for genetic manipulation of the donor organ, potentially enhancing its function. (J Thorac Cardiovasc Surg 1998;115:623-30)

Cardiac gene transfer refers to the alteration of the genetic content of cells within the heart. Typically this is facilitated by the vector-mediated addition of a foreign gene. The further development of such technology might allow novel therapeutic approaches in the treatment of cardiovascular diseases. However, lack of effective and clinically applicable gene delivery systems resulting in high levels of transgene overexpression has been a major obstacle for successful cardiac gene transfer. To date, the adenovirus has proved to be a fairly reliable vector for cardiac gene transfer, because it has the characteristic of being able to infect nondividing cells, which is an absolute requirement for the terminally differentiated myocyte. Direct injection of plasmid deoxyribonucleic acid (DNA) results in localized gene delivery and is not clinically applicable.[1-4] Percutaneous in vivo delivery systems, such as coronary artery catheterization in rabbits[5,6] and

From the Departments of Surgery[a] and Medicine and Biochemistry,[b] Duke University Medical Center, and Howard Hughes Medical Institute,[c] Durham, N.C.

Read at the Seventy-seventh Annual Meeting of The American Association for Thoracic Surgery, Washington, D.C., May 4-7, 1997.

Received for publication May 7, 1997; revisions requested July 15, 1997; revisions received Sept. 29, 1997; accepted for publication Sept. 30, 1997

Address for reprints: Alan P. Kypson, MD, Box 3490, Duke University Medical Center, Durham, NC 27710.

0022-5223/98 $5.00 + 0 12/6/86516

dogs,[7,8] have had variable success, are difficult to reproduce, and infect a limited region of the heart. Ex vivo delivery systems, such as during cardiac transplantation, are clinically relevant and have been shown to allow gene transfer by several different vectors.[9-13]

The application of gene transfer to cardiac transplantation is especially appealing because of the direct access to the donor organ at the time of harvest. One could potentially transfer the foreign genetic material at that time, and expression of the transgene could be evident within 12 to 24 hours. This has been demonstrated in mice, rats, and rabbits.[9-13] Most of these reports however, have focused on the use and overexpression of a marker gene. Because of differences in individual transgene factors such as transgene immunogenicity, the expression of one transgene does not necessarily correlate to the expression of others. Thus in this report we attempted to deliver three different transgenes to the myocardium, including the human β_2-adrenergic receptor (β_2-AR). If myocardium-targeted overexpression of β_2-AR occurs, cardiac function might be enhanced, as has been shown in transgenic mice.[14]

Materials and methods

Construction of recombinant adenovirus. Construction of the cytoplasmic β-galactosidase expressing adenovirus (Ad.LacZ) has been described elsewhere.[15] The *Luciferase* expressing adenovirus (Ad.Luc) was a kind gift from Dr. R. Gerard (University of Texas, Southwestern Medical Center). Construction of the human β_2-adrenergic receptor (Ad.β_2-AR), cell culture conditions, and virus preparation have been described in detail elsewhere.[16]

Animals. All procedures and protocols were approved by the Animal Care and Use Committee of Duke University. Adult male Long Evans rats (250 to 300 gm), obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) were housed under standard conditions and fed a standard diet and water. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Heterotopic heart transplantation. Heterotopic intraabdominal heart transplantation was performed using the technique as previously described by Ono and Lindsey.[17] In brief, both donor and recipient underwent anesthesia with a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) injected intraperitoneally. The donor was heparinized. A clamshell incision was created to expose the thoracic organs. Donor hearts were arrested with 5 ml of normothermic Roe's cardioplegic solution (20 mEq KCl, 27 mEq NaCl, 3 mEq $MgSO_4$, 250 mg methylprednisolone sodium succinate, and 2.25 mEq $NaHCO_3$ for a pH of 7.4). Cardiectomy was performed and 1 ml of adenoviral solution (10^{12} total viral particles) was rapidly injected into the aortic root.

At this point, attention was turned to the recipient. The infrarenal aorta and vena cava were exposed via a midline abdominal incision. An end-to-side anastomosis was performed between the donor and recipient aorta followed by an end-to-side anastomosis of the donor pulmonary artery to the recipient vena cava. Bleeding was controlled with direct pressure. All hearts resumed normal sinus rhythm within 5 minutes. Total ischemic time was between 30 and 50 minutes. During transplantation, the heart was wrapped in gauze and kept at approximately 4° C through use of topical iced saline solution, although myocardial temperature was not measured. After completion of each procedure, the rats recovered under a heat lamp. They were then returned to their cages and allowed free access to food and water. Abdomens were palpated daily to assure a functioning graft.

Experimental design. To assess gene transfer of the marker gene *LacZ*, we gave one group of rats ($n = 6$) the Ad.LacZ solution and another group ($n = 6$) the control solution containing an adenovirus that does not express a transgene (Ad.MT). Both groups of rats were put to death 5 days after transplantation. To assess successful transfer of the *Luciferase* marker gene, we injected one group of rats ($n = 6$) with Ad.Luc and the control group ($n = 6$) with Ad.MT. Both groups were put to death 5 days after transplantation. To determine gene transfer of the β_2-AR, one group ($n = 6$) received Ad.β_2-AR and the controls ($n = 6$) received Ad.MT. Both of these groups were put to death 5 days after transplantation and all hearts were divided into three samples: (1) left and right atria, (2) right ventricle, and (3) left ventricle. Furthermore, groups of three rats each were then infected with Ad.β_2-AR and put to death 3, 5, 7, 10, and 14 days after transplantation. Likewise, all hearts were divided into the previously described three samples.

β-Galactosidase staining. Hearts were excised, rinsed in isotonic saline solution, frozen in a dry ice/isopentane solution, and stored at −80° C. Specimens were mounted on a freezing microtome and 10 μm sections were transferred to glass slides pretreated with aminoalkylsilane (Sigma Chemical Company, St. Louis, Mo.). Sections were fixed in 10% formalin for 2 minutes at room temperature and washed twice in isotonic saline solution. β-Galactosidase staining was carried out in 2 mmol/L $K_4Fe(CN)_6$, 2 mmol/L $K_3Fe(CN)_6$, 2 mmol/L $MgCl_2$, 0.5 mg/ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) in tromethamine-buffered saline solution, pH 7.4. After being stained (usually 30 minutes to 2 hours), the sections were rinsed in tromethamine-buffered saline solution and counterstained with eosin.

***Luciferase* assays.** Hearts were excised, rinsed in isotonic saline solution, and weighed. One milliliter per gram of tissue of 5 mmol/L tromethamine/HCl (pH 7.4), 132 mmol/L NaCl, and 0.5 mmol/L of ethylenediaminetetraacetic acid (EDTA) was added. Specimens were homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y.) and flash-frozen in liquid nitrogen. After one freeze/thaw cycle, the homogenate

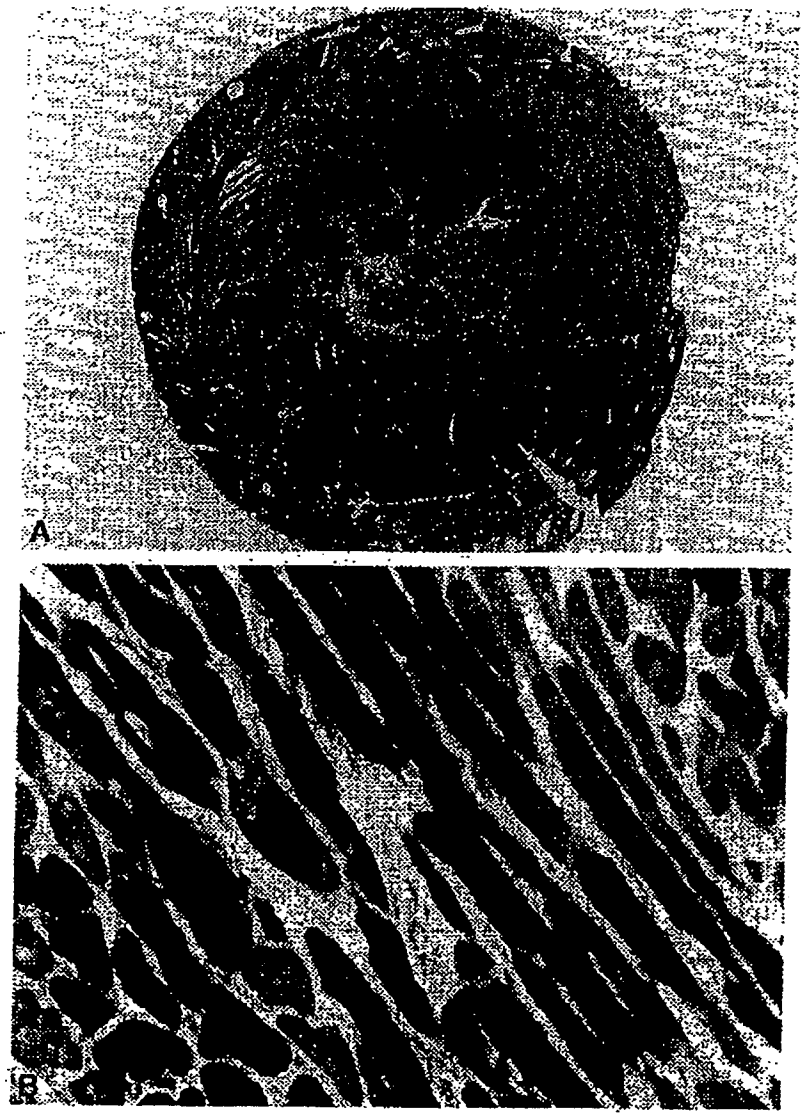


Fig. 1. A, Cross section of a rat heart taken at the mid-ventricular level (2.5× magnification) 5 days after Ad.LacZ infection. B, Same view at higher magnification (100×). Notice individual myocyte staining.

was centrifuged at 10,000g for 2 minutes and the supernatant was saved and assayed for *Luciferase* activity according to the manufacturer's instructions (Promega Corp., Madison, Wis.). In brief, in a 1.5 ml screw-cap Eppendorf tube (Sarsted, Inc., Newton, N.C.), 2 μl of the extract was added to 18 μl of 1x reporter lysis buffer (Promega). Twenty microliters of reconstituted *Luciferase* assay substrate (Promega) was added and incubated for 2 minutes at room temperature. Photon production was then measured in a scintillation counter in single photon counting mode (Beckman Instruments, Inc., Fullerton, Calif.).

β-AR binding assays. Membrane fractions were prepared from hearts and resuspended in binding buffer (75 mmol/L tromethamine-HCl, pH 7.4/12.5 mmol/L $MgCl_2$/2 mmol/L EDTA). Binding assays were performed on 25 μg of membrane protein using saturating amounts of the β-AR–specific ligand [$^{-125}$I]cyanopindolol (300 pmol/L). Nonspecific binding was determined in the presence of 20 μmol/L alprenolol. Reactions were conducted in 500 μl of binding buffer at 37° C for 1 hour and terminated by vacuum filtration through glass-fiber filters. β_1 and β_2 subtype proportions were determined by competition with varying doses of the β_2-selective ligand ICI 118,551. All

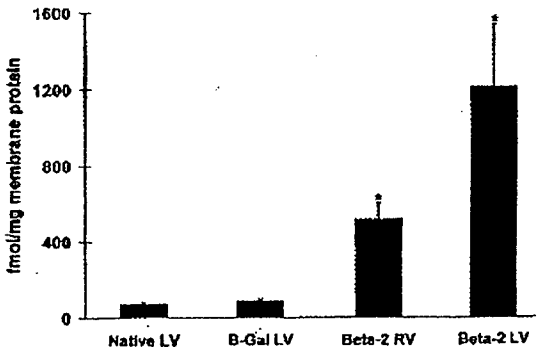


Fig. 2. β-AR density in native recipient hearts ($n = 6$), control transplanted hearts after Ad.LacZ delivery ($n = 6$), and in β_2-AR-treated hearts ($n = 6$) 5 days after transplantation. $^*p < 0.02$ versus controls.

assays were performed in triplicate, and receptor density (fmol) was normalized to milligrams of membrane protein.

Immunohistochemical labeling. Frozen sections were cut at 10 μm for indirect immunofluorescence studies. Sections were rinsed three times for 3 minutes in phosphate-buffered saline solution (PBS) and 3 minutes in PBS with 0.05% octyphenoxy polyethoxyethanol (Triton X-100; Union Carbide Corp., Danbury, Conn.) (Triton-PBS), blocked with serum diluent (10% goat serum in PBS with 0.1% bovine serum albumin and 0.1% sodium azide), and then rinsed for 15 minutes in Triton-PBS before overnight incubation at 4° C with a primary rabbit antihuman β_2-AR antiserum[18] (1:500 dilution in serum diluent). The sections were then washed four times for 10 minutes in Triton-PBS at room temperature and incubated for 1 hour in fluorescein isothiocyanate–conjugated goat antirabbit immunoglobulin G (1:50 dilution in serum diluent). After five 3-minute rinses in PBS, the sections were mounted with sodium iodide (25 gm/L) in 1:1 PBS/glycerol solution and photographed.

Statistical analysis. Quantitative data such as myocardial *Luciferase* activity and β-AR density after adenoviral transgene delivery is expressed as the mean ± standard error of the mean. The difference in the level of transgene expression between control and infected hearts was evaluated with Student's t test.

Results

Survival. Approximately 10% of all rats that underwent transplantation died within 24 hours of the operation. Deaths were equally distributed among the groups. No animals died if they survived the first 24 hours after transplantation. All hearts were in normal sinus rhythm at time of explantation. Histologic examination revealed no abnormalities and differences between the experimental and control groups at all the various times when the animals were put to death.

***LacZ* overexpression.** All hearts that underwent infection with Ad.LacZ demonstrated diffuse staining throughout both ventricles, as well as both atria. Staining was nonuniform and myocytes staining blue were found throughout the various layers of the myocardium, with little evidence of endothelial cell infection (Fig. 1). None of the control hearts stained positive for β-galactosidase.

***Luciferase* overexpression.** All hearts that underwent infection with Ad.Luc demonstrated significant overexpression (970,000 ± 220,000 arbitrary light units) as compared with the controls (500 ± 200 arbitrary light units; $p = 0.001$). Because whole hearts were homogenized for this assay, determination of the distribution of this transgene was not studied.

Human β_2-AR time course of overexpression. Hearts injected with Ad.β_2-AR revealed marked overexpression in both the right and left ventricles as compared with the control hearts (Fig. 2). This represents an approximate sixfold increase in the right ventricle and fourteenfold increase in the left ventricle. The fraction of β_2-ARs increased to 90% in the left ventricle from a fraction of 30% in the control hearts, demonstrating that the increased β-AR density was due exclusively to β_2-AR overexpression. Furthermore, immunohistochemical analysis for the human β_2-AR revealed several areas of diffuse staining of myocardial sarcolemmal membranes of various intensities (Fig. 3).

In rats put to death 3 to 14 days after transplantation, peak overexpression occurred at 5 days, and β-AR density was back to control values by 14 days, although at 10 days overexpression was still significant. The atria exhibited the same temporal pattern of overexpression as the left ventricle, whereas overexpression in the right ventricle peaked at 3 days and remained at that level for the first 7 days before returning to control values by postoperative day 14 (Fig. 4).

Discussion

The milieu of the heart during transplantation is an ideal situation for the introduction of foreign genetic material into cardiac myocytes. Gene transfer to the donor organ, at the time of harvest, can easily be accomplished with an adenoviral vector. The role of gene transfer for the treatment of cardiac disorders is evolving and may potentially

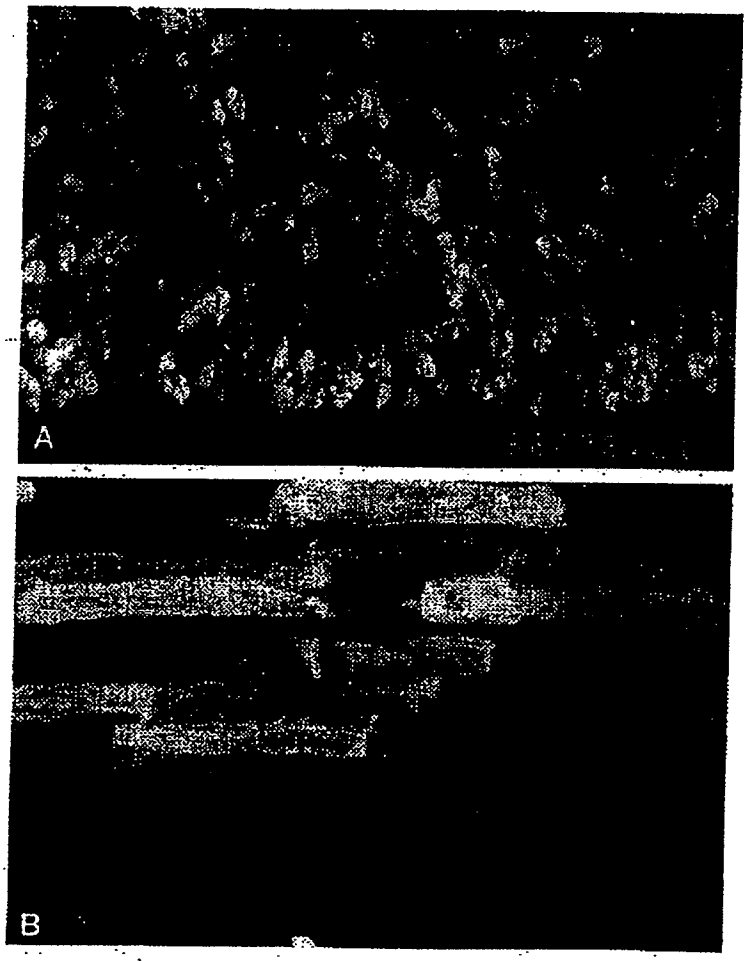


Fig. 3. Representative immunohistochemical detection of expressed human β_2-ARs 5 days after Ad.β_2-AR delivery to transplanted rat hearts. **A**, Cross-sectioned myocytes at 25× magnification. **B**, Longitudinally sectioned myocytes at 100× magnification.

include applications for the treatment of congestive heart failure, transplant rejection and dysfunction, and ischemic heart disease. However, it remains to be seen whether different transgenes that would be required to treat these disorders can be directed to and overexpressed in the human myocardium under the conditions of current technology.

Recently, the adenovirus vector has been shown to have increased efficacy of gene transfer into cells both in vivo and in vitro.[6, 11, 12, 16] Recombinant adenoviral vectors have a number of advantages over other viral and nonviral vectors. Adenoviral vectors provide efficient transfer into cells that are terminally differentiated. They can be made replication deficient, can be produced in high titer, and can carry up to 7.5 kb of exogenous genetic material.[19] Therefore this study took advantage of these characteristics to test delivery of three different transgenes, one with a potential future application of improving myocardial contractility.

The present study demonstrates that the time of cardiac transplantation is an ideal setting for the administration of an adenoviral solution to "genetically modulate" the organ. Several reports have shown that the expression of reporter genes directly injected into the myocardium is limited to a finite area around the site of injection and is further complicated by local inflammation.[1, 3] Therefore this raises doubts about the clinical applicability of this method of gene delivery. In vivo intracoronary

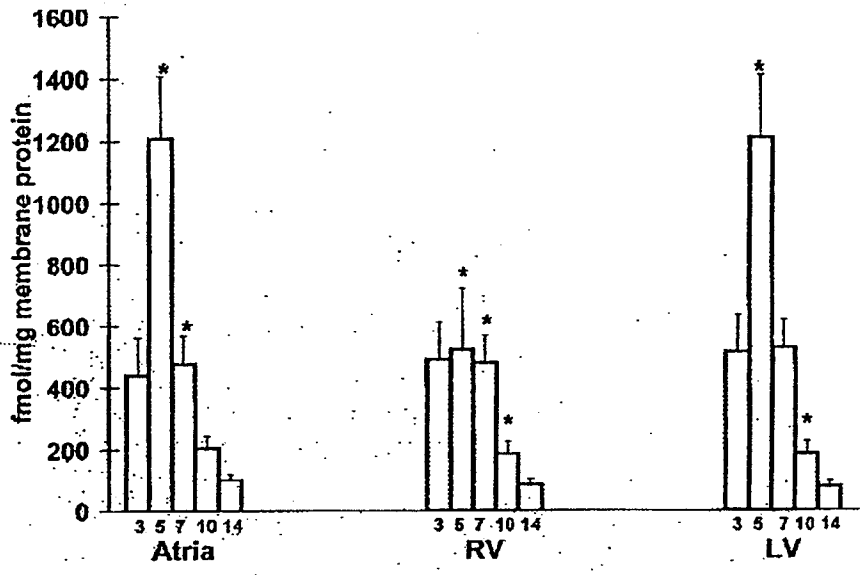


Fig. 4. Time course of overexpression at 3, 5, 7, 10, and 14 days of β-ARs in atria, right ventricle *(RV)*, and left ventricle *(LV)* after delivery of 10^{12} total viral particles of Ad.β_2-AR ($n = 3$ per time point). $^*p < 0.05$ compared with β-AR density of control native hearts (75.6 ± 6.4).

delivery has been demonstrated,[5, 6] but it is very difficult to reproduce consistently, probably because of insufficient contact time between the adenovirus and the beating myocardium, as well as a host of other reasons that have yet to be identified.

Unlike direct injection, infection through the coronary vasculature during cardiac transplantation results in robust and widespread overexpression of the transgene, as evidenced by the extensive β-galactosidase staining of the right and left ventricles on histologic examination, as well as the marked overexpression of the β_2-AR, not only in both ventricles, but in both atria as well. The transplantation model is unique in that it provides the mechanism to accomplish global myocardial gene transfer. First, adenoviral solution is injected into the aortic root, presumably via both right and left coronary arteries, providing access to the entire myocardium. The solution is delivered immediately after removal of the heart at normothermia. No hypothermia is used in this model because this may reduce the efficacy of gene transfer by slowing the kinetics of the virus-receptor interaction. In fact, we have observed lower amounts of adenoviral gene transfer with hypothermia (data not shown). Furthermore, donor heart ischemia may facilitate viral transfection by making the endothelium more permeable. This, combined with the fact that the adenoviral solution is in constant contact with the myocardium throughout the procedure, likely further enhances gene transfer across the endothelium and into the myocardium itself. This constant exposure probably saturates the viral receptors and must partially account for the robust overexpression of transgenes seen in this model. As opposed to other reports,[9, 11, 13] we have not observed any significant expression in endothelial cells within the vasculature of the heart, but extensive gene transfer to myocytes was evidenced by the histologic pattern of *LacZ* staining, as well as the pattern of β_2-AR overexpression detected by immunohistochemistry. The lack of endothelial expression was surprising, and further studies will be required to shed more light on the mechanism of myocyte/endothelial cell gene transfer in our model. Ultimately, however, enhancement of cardiac function through β-AR overexpression requires targeting myocytes rather than endothelial cells, which has been achieved in this report.

The analysis of the time course of gene expression in our model correlates fairly closely with other reports.[11, 12] Peak β_2-AR overexpression was reached at 5 days, and by 14 days expression was back to control values. However, we have no explanation for the apparent difference in the kinetics of transgene expression in the right ventricle (Fig. 4). More recently, we have looked at expression at 28

days and have found no evidence of significant overexpression. Some reports[20] describe stable gene expression up to months, but these are in different animal models with different delivery systems. Clearly, further work will have to be done to increase the stability of these transgenes. Probable advances will come in work done on the adenovirus itself by further deleting immunogenic regions, in essence "hiding" the adenovirus from the host's immune system. An immune response to adenovirus has been reported[1, 21] and may be partly responsible for the transient overexpression seen with this model. Of note, immunosuppressive therapies have been shown to greatly extend the duration of transgene expression mediated by recombinant replication-deficient adenovirus.[22, 23] Although we have not yet examined the role of immunosuppression in our model, it is possible that the immunosuppressive regimen used currently in the cardiac posttransplant setting might allow for extended transgene expression mediated by these viruses. However, this may also prove to make the recipient more vulnerable to potential adenoviral infections using the current vectors. The creation of advanced gene transfer vectors, which is the focus of several groups, should eventually eliminate this possibility. Importantly, in our model, there was no histologic evidence in the transplanted rat hearts of any direct injury caused by the adenovirus out to 28 days after transplantation.

In conclusion, we have shown that ex vivo adenovirus-mediated gene transfer is possible in the adult rat heterotopic heart transplant model. Not only have we demonstrated successful gene transfer of two marker genes, but we have also demonstrated for the first time robust and global overexpression of a gene encoding for an endogenous membrane protein (β_2-AR), which has been shown to increase myocardial contractility in transgenic mice.[14] Thus, in future studies, it will be critical to document the functional significance of β_2-AR overexpression in this model to determine whether genetic modification can be successful at improving myocardial function in the posttransplantation setting.

REFERENCES

1. Acsadi G, Jiao S, Jani A, Duke D, Williams P, Chong W, et al. Direct gene transfer and expression into rat heart in vivo. New Biol 1991;3:71-81.
2. Buttrick PM, Kass A, Kitsis RN, Kaplan ML, Leinwand LA. Behavior of genes directly injected into the rat heart in vivo. Circ Res 1992;70:193-8.
3. Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 1990;82:2217-21.
4. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circ Res 1993;73:1202-7.
5. Barr E, Carroll J, Kalynych AM, Tripathy SK, Kozarsky K, Wilson JM, et al. Efficient catheter-mediated gene transfer into the heart using replication-defective adenovirus. Gene Ther 1994;1:51-8.
6. Lilly RE, Peppel K, Kypson AP, Silvestry SC, Glower, DD, Koch WJ, et al. Intracoronary administration of adenovirus for gene transfer into adult rabbit myocardium. Surg Form 1996;47:279-81.
7. Chapman GD, Lim CS, Gammon RS, Culp SC, Desper S, Bauman RP, et al. Gene transfer into coronary arteries of intact animals with a percutaneous balloon catheter. Circ Res 1992;71:27-33.
8. Magovern CJ, Mack CA, Zhang J, Hahn RT, Ko W, Isom OW, et al. Direct in vivo gene transfer to canine myocardium using a replication-deficient adenovirus vector. Ann Thorac Surg 1996;62:425-34.
9. Dalesandro J, Akimoto H, Gorman CM, McDonald TO, Thomas R, Liggitt HD, et al. Gene therapy for donor hearts: ex vivo liposome-mediated transfection. J Thorac Cardiovasc Surg 1996;111:416-21.
10. Ardehali A, Fyfe A, Laks H, Drinkwater DC Jr, Qiao JH, Lusis AJ. Direct gene transfer into donor hearts at the time of harvest. J Thorac Cardiovasc Surg 1995;109:716-20.
11. Lee J, Laks H, Drinkwater DC, Blitz A, Lam L, Shiraishi Y, et al. Cardiac gene transfer by intracoronary infusion of adenovirus vector-mediated reporter gene in the transplanted mouse heart. J Thorac Cardiovasc Surg 1996;111:246-52.
12. Wang J, Ma Y, Knechtle, SJ. Adenovirus-mediated gene transfer into rat cardiac allografts. Transplantation 1996;61:1726-9.
13. Sawa Y, Suzuki K, Bai HZ, Shirakura R, Morishita R, Kaneda Y, et al. Efficiency of in vivo gene transfection into transplanted rat heart by coronary infusion of HVJ liposome. Circulation 1995;92(Suppl):II479-82.
14. Milano CA, Allen LF, Rockman HA, Dolber PC, McMinn TR, Chien KR, et al. Enhanced myocardial function in transgenic mice overexpressing the β_2-adrenergic receptor. Science 1994;264:582-6.
15. Kolls JK, Peppel K, Silva M, Beutler B. Prolonged and effective blockade of tumor necrosis factor activity through adenovirus-mediated gene transfer. Proc Natl Acad Sci U S A 1994;91:215-9.
16. Drazner MH, Peppel K, Dyer S, Grant AO, Koch WJ, Lefkowitz RJ. Potentiation of adrenergic signaling by adenoviral-mediated gene transfer in adult rabbit ventricular myocytes. J Clin Invest 1997;99:288-96.
17. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225-9.
18. von Zastrow M, Kobilka BK. Ligand-regulated internalization and recycling of human beta$_2$-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. J Biol Chem 1992;267:3530-8.
19. Berkner KL. Development of adenovirus vectors for expression of heterogeneous genes. Biotechniques 1988;6:616-29.
20. Nabel EG, Pautz G, Nabel GY. Gene transfer into vascular cell. J Am Coll Cardiol 1991;17:189B-94B.

21. Yang Y, Ertl HCJ, Wilson JM. MHC class I–restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1994;1:433-42.
22. Lochmuller H, Petrof BJ, Pari G, Larochelle N, Dodelet V, Wang Q, et al. Transient immunosuppression by FK506 permits a sustained high level dystrophin expression after adenoviral-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice. Gene Ther 1996;3: 706-16.
23. Kass-Eisler A, Leinwald L, Gall J, Bloom B, Falck-Pedersen E. Circumventing the immune response to adenovirus-mediated gene therapy. Gene Ther 1996;3:154-62.

Discussion

Mr. Magdi H. Yacoub *(London, United Kingdom).* Have you looked at the effect of overexpression of β-receptors on function of these cells in any way, either in vitro or in any other way?

Dr. Alan P. Kypson *(Durham, N.C.).* Yes, we have just started our preliminary studies.

We transfected six hearts with the β_2-receptor and showed overexpression. (Average β-receptor density was 400 fmol as compared our controls [80 fmol]). These same rat hearts were then mounted on a Langendorff apparatus 5 days after infection and there was almost a doubling of the baseline rate of pressure rise in these infected hearts as compared with the control hearts. We have yet to do β-agonist dose/response curves as well as evaluate the amount of overexpression correlating with the functional effect. These are very preliminary data, but there does seem to be some sort of a functional enhancement.

Dr. Tirone E. David *(Toronto, Ontario, Canada).* These measurements are done at what stage of the experiment?

Dr. Kypson. At 5 days we remove the heart from the abdomen and perfuse it on a Langendorff apparatus.

Dr. David. Did you do any after 14 days?

Dr. Kypson. No. We have looked only at 5 days so far.

Mr. John H. Kennedy *(Cambridge, United Kingdom).* Is the life of the transfected cells shorter than the life of the host or longer? They are obviously better.

Dr. Kypson. There are some who believe that the cells that overexpress these transgenes undergo an immune response by the host. We have looked at the pathology of these hearts 28 days after transplantation and have seen normal histologic characteristics.

Dr. D. Glenn Pennington *(Winston-Salem, N.C.).* Is there a real difference in the right and the left ventricles, or is that just a matter of muscle mass?

Dr. Kypson. Actually, this probably relates to some sort of an increased transmural pressure wall gradient generated in the left ventricle, which enhances transduction of the viral particles across the endothelium into the myocardium.

EXHIBIT S

C17

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 5251–5256, April 1998
Medical Sciences

Modulation of ventricular function through gene transfer *in vivo*

(sarcoplasmic reticulum/phospholamban/calcium/Ca^{2+} ATPase/adenovirus)

ROGER J. HAJJAR, ULRICH SCHMIDT, TAKASHI MATSUI, J. LUIS GUERRERO, KYUNG-HAN LEE, JUDITH K. GWATHMEY*, G. WILLIAM DEC, MARC J. SEMIGRAN, AND ANTHONY ROSENZWEIG†

Cardiovascular Research Center and Heart Failure and Cardiac Transplantation Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129; and *Boston University School of Medicine, Boston, MA 02118

Communicated by Alexander Leaf, Massachusetts General Hospital, Charlestown, MA, February 26, 1998 (received for review November 27, 1997)

ABSTRACT We used a catheter-based technique to achieve generalized cardiac gene transfer *in vivo* and to alter cardiac function by overexpressing phospholamban (PL) which regulates the activity of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a). By using this approach, rat hearts were transduced *in vivo* with 5×10^9 pfu of recombinant adenoviral vectors carrying cDNA for either PL, β-galactosidase (β-gal), or modified green fluorescent protein (EGFP). Western blot analysis of ventricles obtained from rats transduced by Ad.PL showed a 2.8-fold increase in PL compared with hearts transduced by Ad.βgal. Two days after infection, rat hearts transduced with Ad.PL had lower peak left ventricular pressure (58.3 ± 12.9 mmHg, $n = 8$) compared with uninfected hearts (92.5 ± 3.5 mmHg, $n = 6$) or hearts infected with Ad.βgal (92.6 ± 5.9 mmHg, $n = 6$). Both peak rate of pressure rise and pressure fall (+3, 210 ± 298 mmHg/s, −2, 117 ± 178 mmHg/s, $n = 8$) were decreased in hearts overexpressing PL compared with uninfected hearts (+5, 225 ± 136 mmHg/s, −3, 805 ± 97 mmHg/s, $n = 6$) or hearts infected with Ad.βgal (+5, 108 ± 167 mmHg/s, −3, 765 ± 121 mmHg/s, $n = 6$). The time constant of left ventricular relaxation increased significantly in hearts overexpressing PL (33.4 ± 3.2 ms, $n = 8$) compared with uninfected hearts (18.5 ± 1.0 ms, $n = 6$) or hearts infected with Ad.βgal (20.8 ± 2.1 ms, $n = 6$). These differences in ventricular function were maintained 7 days after infection. These studies open the prospect of using somatic gene transfer to modulate overall cardiac function *in vivo* for either experimental or therapeutic applications.

The regulation of intracellular calcium is intimately related to the systolic and diastolic function of cardiac cells (1, 2). The sarcoplasmic reticulum (SR), which releases calcium during systole and takes it up during diastole, plays an integral part in controlling the synchronized movement of calcium in myocardial cells. The SR Ca^{2+} ATPase (SERCA2a) pump regulates the uptake of Ca^{2+} into the SR during diastole. The function of the SERCA2a pump is regulated in turn by phospholamban (PL) (3). In its unphosphorylated form, PL inhibits the SERCA2a pump whereas in its phosphorylated form, this inhibition is relieved. A decrease in SERCA2a activity has been identified in a number of animal models of heart failure and in human heart failure and an increase in the relative ratio of PL to SERCA2a appears to be an important characteristic of both experimental and human heart failure (3, 4). We have previously modeled such alteration in the PL/SERCA2a ratio by using adenoviral gene transfer to cardiocytes *in vitro*. Adenoviral overexpression of PL *in vitro* recapitulates many of the physiological abnormalities seen in heart failure, including prolonged relaxation and decreased contractile function. In contrast, overexpression of SERCA2a enhances relaxation and contractility of normal cardiomyocytes and rescues myocytes overexpressing PL from their abnormal phenotype (5, 6). Cardiac gene transfer has been previously achieved predominantly by direct injection into the myocardium or perfusion of an isolated coronary segment (7, 8). Either approach results in focal overexpression of the transgene and is therefore unlikely to effectively modulate global cardiac function. In this study we used a catheter-based technique to achieve highly effective transgene expression in rat heart *in vivo*. *In vivo* overexpression of PL resulted in profound physiological alterations in cardiac function including a decrease in left ventricular systolic pressure and an increase in diastolic pressure and a prolonged isovolumic relaxation. As in single cells overexpressing PL, these effects mimic abnormalities seen in experimental and human heart failure. Our data suggest that global adenoviral gene transfer to rodent hearts *in vivo* may be a useful tool for studying the molecular mechanisms regulating cardiac function. Overexpression of PL in particular creates an acquired phenotype that recapitulates many abnormalities seen in human heart failure and may provide a useful model for testing therapeutic interventions.

Materials & Methods

Construction of E1-Deleted Recombinant Adenoviral Vectors. Three first generation type 5 recombinant adenoviruses (Ad) were used in these studies: Ad.βgal, Ad.PL, and Ad.EGFP. Ad.βgal, which carries a nuclear localizing form of β-galactosidase (β-gal), utilizes the dl327 backbone and was kindly provided by David Dichek (Gladstone Institute for Cardiovascular Diseases, San Francisco, CA) (9). The construction of Ad.PL has been described in detail (6). Ad.EGFP was similarly constructed through homologous recombination in 293 cells by using pJM17 as a source of adenoviral DNA. Ad.EGFP carries the cDNA for modified green fluorescence protein, EGFP, purchased from Clontech. In each, the exogenous cDNA has been substituted for E1 through homologous recombination in 293 cells and each contains a small deletion in E3. The recombinant viruses were prepared as high titer stocks by propagation in 293 cells as described (5, 6). The titer of stocks used for these studies measured by plaque assays were as follows: 3.1×10^{10} pfu/ml for Ad.PL, 2.7×10^{10} pfu/ml for Ad.βgal, and 2×10^{10} pfu/ml for Ad.EGFP with a particle/pfu ratio of 40:1, 37:1, and 50:1, respectively (viral particles/ml determined by using the relationship one absorbance unit at 260 nm is equal to 10^{12} viral particles/ml).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PL, phospholamban; EGFP, modified green fluorescent protein; SR, sarcoplasmic reticulum; SERCA2a, SR Ca^{2+} ATPase; Ad, adenovirus; β-gal, β-galactosidase; LVSP, left ventricular systolic pressure.

†To whom reprint requests should be addressed at: Cardiovascular Research Center, Massachusetts General Hospital, East, 149 13th Street, 4th Floor, Room 4207, Charlestown, MA 02129.

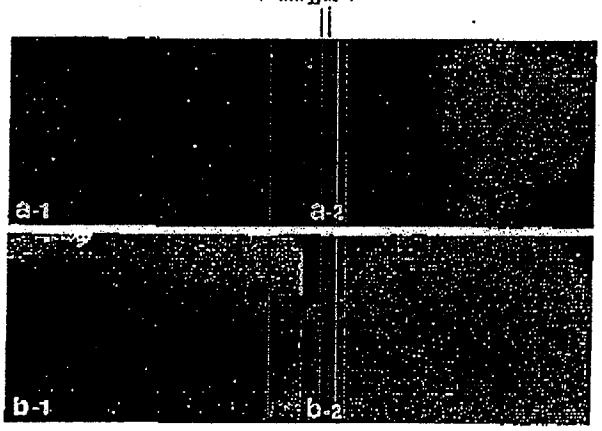


FIG. 1. Rat hearts were transduced with Ad.EGFP by using either the catheter-based technique (*b*) or direct injection into the left ventricular wall (*a*). Forty-eight hours following delivery of adenovirus encoding for EGFP, the left ventricles of the hearts were removed and visualized with white light (*a-1* and *b-1*) and at 510 nm with single excitation peak at 490 nm of blue light (*a-2* and *b-2*). As shown in *b-2*, the expression pattern observed after catheter delivery is grossly homogeneous. In contrast, the expression pattern is localized after direct injection as shown in *a-2*. Of note, with the direct injection, the surrounding tissue exhibits no background fluorescence (*a-2*).

Adenoviral Delivery Protocol. Rats were anesthetized with pentobarbital i.p. and placed on a ventilator. The chest was entered from the left side through the third intercostal space. The pericardium was opened and a 7-0 suture placed at the apex of the left ventricle. The aorta and pulmonary artery were identified. A 22 G catheter containing 200 μl of adenovirus was advanced from the apex of the left ventricle to the aortic root. The aorta and pulmonary arteries were clamped distal to the site of the catheter and the solution injected. The clamp was maintained for 10 s when the heart pumped against a closed system (isovolumically). This procedure allows the solution that contains the adenovirus to circulate down the coronary arteries and perfuse the heart without direct manipulation of the coronaries. After 10 s, the clamp on the aorta and pulmonary artery was released. After removal of air and blood, the chest was closed, and animals were extubated and transferred back to their cages.

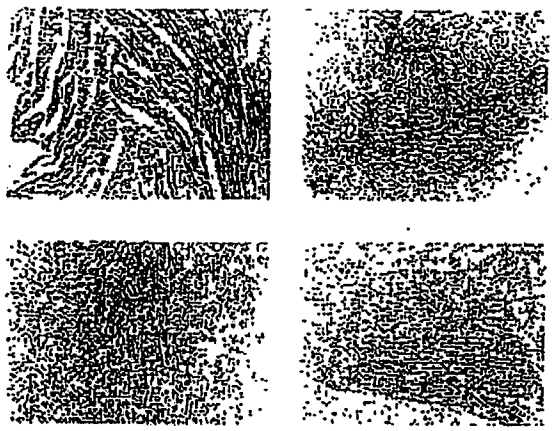


FIG. 2. Expression of β-gal in left ventricular sections 2 days following infection with Ad.βgal and Ad.PL. (*Upper*) Photomicrographs of two left ventricular sections stained for β-gal 2 days following infection with Ad.βgal. These sections show the variability of β-gal expression within the same heart with the catheter-based method of gene delivery. (*Lower*) Photomicrographs of two left ventricular sections stained for β-gal 2 days following infection with Ad.PL. No β-gal expression is observed.

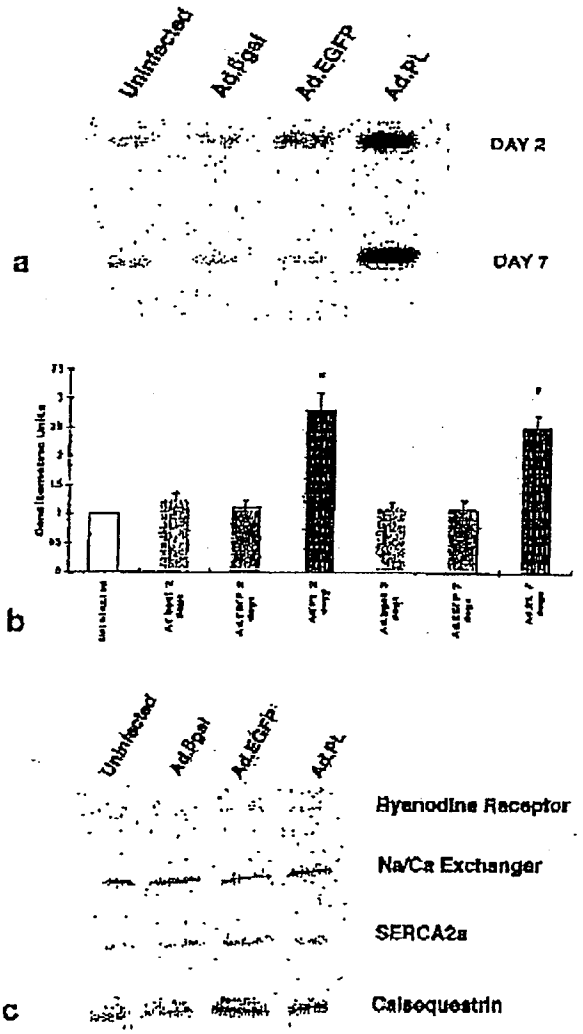


FIG. 3. (*a*) Immunoblots of PL from crude membranes of left ventricles from control uninfected rats or rats 2 and 7 days after infection with 5×10^9 pfu of either Ad.βgal, Ad.EGFP, or Ad.RSV.PL. (*b*) Protein levels of PL in preparations from uninfected hearts ($n = 8$), preparations of hearts infected with Ad.βgal at day 2 ($n = 6$), preparations of hearts infected with Ad.EGFP at day 2 ($n = 4$), preparations of hearts infected with Ad.PL at day 2 ($n = 8$), preparations of hearts infected with Ad.βgal at day 7 ($n = 6$), preparations of hearts infected with Ad.EGFP at day 7 ($n = 4$), and preparations of hearts infected with Ad.PL at day 7 ($n = 8$). *, $P < 0.05$ compared with uninfected, Ad.βgal at 2 days, and Ad.EGFP at 2 days. #, $P < 0.05$ compared with Ad.βgal at 2 days, and Ad.EGFP at 7 days. There were no significant differences between the PL protein levels in the uninfected group and Ad.βgal or Ad.EGFP ($P > 0.2$). (*c*) Immunoblots with mAbs to the of ryanodine receptor, Na/Ca exchanger, SERCA2a, and calsequestrin from crude membrane of left ventricles after 2 days of infection with 5×10^9 pfu of either Ad.βgal, Ad.EGFP, or Ad.RSV.PL.

Pressure Measurements. Rats in the different treatment groups and at different stages following adenoviral gene transfer were anesthetized with 60 mg/kg of pentobarbital and mechanically ventilated. The chest was then opened through a mid-line incision and the heart exposed. A small incision was then made in the apex of the left ventricle and a 2.0 French high fidelity pressure transducer (Millar Instruments, Houston, TX) introduced into the left ventricle. Pressure measurements were digitized at 1 KHz and stored for further analysis. Left

Table 1. Systolic and diastolic parameters

	HR, min^{-1}	LVSP, mmHg	LVDP, mmHg	+dP/dt, mmHg/sec	−dP/dt, mmHg/sec	τ, msec	n
Uninfected	302 ± 21	92.5 ± 3.5	5.2 ± 1.1	5,225 ± 136	−3,805 ± 97	18.5 ± 1.0	6
Ad.βgal day 2	286 ± 19	92.6 ± 5.9	6.3 ± 1.6	5,108 ± 167	−3,765 ± 121	20.8 ± 2.1	6
Ad.PL day 2	270 ± 26	58.3 ± 12.9*	12.7 ± 2.9*	3,210 ± 298*	−2,117 ± 178*	33.4 ± 3.2*	8
Ad.βgal day 7	290 ± 22	88.5 ± 5.0	6.5 ± 1.3	5,032 ± 234	−3,668 ± 112	21.1 ± 2.4	6
Ad.PL day 7	342 ± 31	54.2 ± 8.2†	9.8 ± 2.3	3,345 ± 311†	−2,345 ± 154†	32.4 ± 1.9†	8

All data presented as mean ± SD. LVSP, left ventricular systolic pressure; LVDP, left ventricular end-diastolic pressure; HR, heart rate; +dP/dt, maximal rate of pressure rise; −dP/dt, maximal rate of pressure fall; τ, time constant of relaxation; n, number of hearts.
*$P < 0.01$ compared to uninfected and Ad.βgal, day 2.
†$P < 0.01$ compared to Ad.βgal, day 7.

ventricular systolic pressure (LVSP), end-diastolic left ventricular pressure (LVDP), the maximal rates of pressure rise (+dP/dt) and of pressure fall (−dP/dt), and the time constant of relaxation (τ) were measured or derived in the different groups. The time course of isovolumic relaxation was measured by using the equation: $P = P_0 e^{-t/\tau} + P_B$, where P is the left ventricular isovolumic pressure, P_0 is pressure at the time of peak −dP/dt, and P_B is residual pressure.

Left Ventricular Dimension Measurements. Multiple 1.3-mm piezoelectric crystals (Sonometrics, Alberta, Canada) were placed over the surface of the left ventricle along the short axis of the ventricle at the level of the mitral valve. The intercrystal distance was recorded along with the left ventricular pressure. Left ventricular pressure dimension loops were generated under different loading conditions by clamping the inferior vena cava. The end-systolic pressure–dimension relationship was obtained by producing a series of pressure dimension loops over a range of loading conditions and connecting the upper left hand corners of the individual pressure dimension loops to generate the maximal slope (E_{max}).

Preparation of SR Membranes from Left Ventricles. To isolate SR membrane from hearts, we used a procedure modified from Harigaya and Schwartz (10). Briefly, left ventricular myocardium was suspended in a buffer containing 300 mmol/liter sucrose, 1 mmol/liter phenylmethylsulfonyl fluoride,and 20 mmol/liter Pipes (pH 7.4). The tissue was then disrupted with a homogenizer. The homogenates were centrifuged at 500 × *g* for 20 min. The resultant supernatant was centrifuged at 25,000 × *g* for 60 min to pellet the SR-enriched membrane. The pellet was resuspended in a buffer containing 600 mmol/liter KCl, 30 mmol/liter sucrose, and 20 mmol/liter Pipes, frozen in liquid nitrogen and stored at −70°C. Protein concentration was determined in these preparations by a modified Bradford procedure (11) with BSA for the standard curve (Bio-Rad).

Histochemistry. Hearts were examined by immunohistochemistry to evaluate the expression of β-galactosidase. Hearts were fixed with PBS containing 0.5% glutaraldehyde for 30 min and then in PBS with 30% sucrose for 30 min. The hearts were then permeabilized by incubation in solution containing sodium deoxycholate (0.01%) and Nonidet P-40 for 15 min. Then the hearts were incubated overnight in a solution containing 5-bromo-4-chloro3-indolyl β-D-galactopyranoside (X-Gal), and 10-μm sections were then cut and examined under light microscopy. Lungs, livers, and full-length aorta were also fixed and examined in a similar fashion.

Western Blot Analysis of PL and SERCA2a in SR Preparations. SDS/PAGE was performed on the isolated membranes from cell cultures under reducing conditions on a 7.5% separation gel with a 4% stacking gel in a Miniprotean II cell (Bio-Rad). Proteins were then transferred to a Hybond-ECL nitrocellulose for 2 h. The blots were blocked in 5% nonfat milk in TRIS-buffered saline for 3 h at room temperature. For immunoreaction, the blot was incubated with 1:2,500 diluted mAbs to either SERCA2a, Na/Ca exchanger, ryanodine receptors, and calsequestrin (Affinity BioReagents, Golden, CO) or 1:2,500 diluted anti-cardiac PL monoclonal IgG (Upstate Biotechnology, Lake Placid, NY) for 90 min at room temperature. After washing, the blots were incubated in a solution containing peroxidase-labeled goat anti-mouse IgG (dilution 1:1000) for 90 min at room temperature. The blot was then incubated in a chemiluminescence system and exposed to an X-Omat x-ray film (Fuji Films) for 1 min. The densities of the bands were evaluated by using NIH IMAGE. Normalization was performed by dividing densitometric units of each membrane preparation by the protein amounts in each of these preparations. Serial dilution of the membrane preparations revealed a linear relationship between amounts of protein and the densities of the PL immunoreactive bands (data not shown).

RESULTS

Cardiac Gene Transfer. *In vivo* cardiac gene transfer was achieved by using a catheter inserted at the left ventricular apex and terminating just above the aortic valve. The adenoviral preparation was injected as the aorta and pulmonary artery were cross-clamped distal to the catheter tip for 10 s. During this period, the right and left ventricles became visibly pale as clear viral solution perfused the myocardium through the coronary arteries. During the procedure, heart rate decreased from ≈300 beat per minute (bpm) to ≈50 bpm, but recovered to baseline within 30 s of clamp release. Left ventricular systolic pressure increased to ≈300 mmHg and diastolic pressure to ≈25 mmHg. Ventricular pressure returned to baseline within 60 s of releasing the clamp. To examine the distribution of transgene expression, we used two adenoviruses carrying the reporter genes β-gal and EGFP, Ad.βgal and Ad.EGFP, respectively. As shown in Fig. 1, 2 days following delivery of Ad.EGFP with the catheter-based technique, the expression pattern observed was grossly homogeneous. In contrast, when Ad.EGFP was directly injected into the left ventricular wall the expression pattern was localized, whereas the surrounding tissue exhibited no background fluorescence.

Hearts were also examined with immunohistochemistry to evaluate the microscopic distribution of transgene expression *in vivo*. As shown in Fig. 2, histochemical staining of ventricular cross-sections from hearts infected with Ad.βgal (day 2) revealed β-gal activity in myocytes not observed in Ad.PL-infected hearts. The distribution of β-gal was not uniform in all cross-sections. Certain areas had diffuse staining, whereas other sections had a more patchy distribution of expression. It is important to note that we used an adenovirus carrying a nuclear localized form of β-gala. Cytoplasmic β-gal activity is evident only in myocytes expressing the highest level of β-gal activity. Therefore, a ventricular section that typically reveals a small minority of muscle nuclei may underestimate the uniformity and the level of nuclear β-gal expression. To evaluate whether other tissues are infected, we histologically examined sections of aorta, liver, and lung following infection

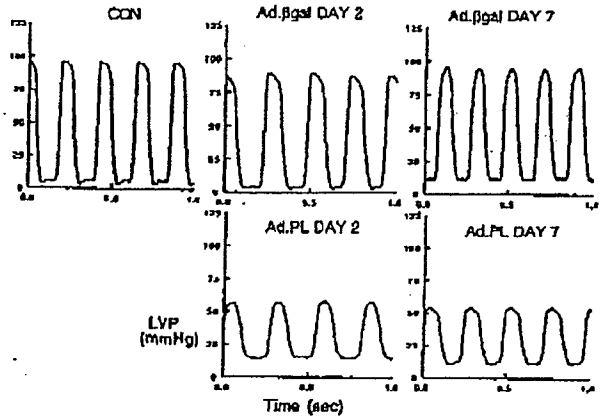


FIG. 4. Left ventricular pressure measurements from rats that were either uninfected (CON) or infected with Ad.βgal or Ad.PL 2 and 7 days after infection as indicated. Hearts infected with Ad.PL had a decrease in systolic pressure, elevation of diastolic pressure, and prolongation of the relaxation phase.

with Ad.βgal. There were no histological evidence of β-gal activity in the aorta; however, β-gal activity was present in the liver and lungs (data not shown). Infected rat hearts demonstrated an inflammatory response (7 days > 2 days postinfection); however, there was no evidence of disruption of normal myocardial architecture or collagen deposition.

PL Overexpression. By using the technique described above, we transduced rat hearts with an adenovirus carrying PL (Ad.PL). As shown in Fig. 3*a*, infection with Ad.βgal or EGFP did not significantly alter the amount of PL in the rat hearts, whereas infection with Ad.PL increased the amount of PL by ≈2.5- to 3-fold. The increase in PL was also sustained at day 7. Quantification by densitometry of the immunoblots, depicted in Fig. 3*b*, shows an increase in PL at 2 days and at 7 days when compared with uninfected hearts. To verify that overexpression of PL *in vivo* does not affect other proteins involved in maintaining intracellular calcium homeostasis, Western blot analysis was performed for SERCA2a, the ryanodine receptor, calsequestrin, and the Na/Ca exchanger. As shown in Fig. 3*c*, infection with Ad.βgal, Ad.EGFP, or Ad.PL did not affect the level of these proteins.

Effects of PL Overexpression on Hemodynamic Measurements. We next examined the physiological consequences of adenoviral gene transfer of β-gal or PL to rat hearts *in vivo*. As shown in Table 1, β-gal infection did not alter heart rate or any of the hemodynamic parameters examined compared with uninfected hearts at day 2 or day 7. Infection with Ad.PL did not significantly change heart rate at 2 days; however, at 7 days following infection with Ad.PL there was a trend toward an increase in heart rate. As shown in Fig. 4 and Table 1, the LVSP was significantly decreased in hearts overexpressing PL at both 2 and 7 days following infection, whereas left ventricular diastolic pressure (LVDP) was significantly increased in these hearts when compared with uninfected controls and to hearts infected with Ad.βgal. The peak rate of pressure rise (+dP/dt), a reflection of systolic function, was significantly decreased in hearts overexpressing PL at both 2 and 7 days. The peak rate of pressure fall, an index of diastolic function, was significantly decreased in hearts overexpressing PL compared with uninfected or Ad.βgal-infected hearts. The time constant of relaxation, which is an index of active relaxation, was significantly prolonged in hearts overexpressing PL compared with uninfected or Ad.βgal-infected hearts.

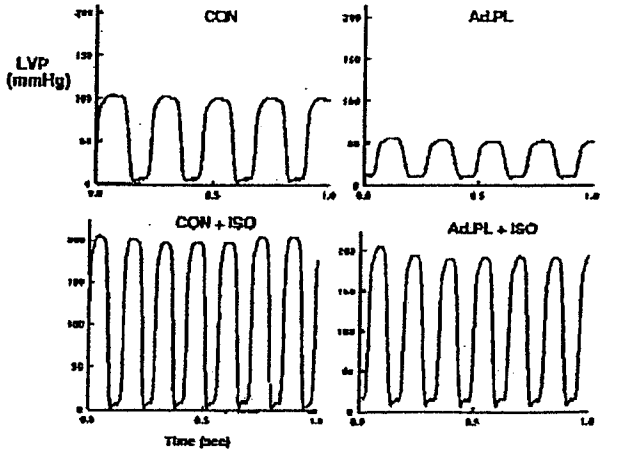


FIG. 5. Left ventricular pressure measurements during infusion of 0.1 μg/kg/min of isoproterenol in an uninfected rat heart and rat hearts infected with Ad.PL (5×10^9 pfu, day 2).

Effect of Isoproterenol. To verify the specificity of the profound hemodynamic effects observed after PL overexpression, we pharmacologically inhibited PL with isoproterenol, inducing the phosphorylation of PL thereby removing its inhibition of SERCA2a. The ventricular function after isoproterenol reflects the intrinsic SERCA2a activity. Therefore, induction of PL phosphorylation by isoproterenol should result in the same hemodynamic profile in both Ad.PL and uninfected hearts, reflecting their similar and unaffected intrinsic SR ATPase activity. As seen in Fig. 5, isoproterenol increased LVSP and relaxation in hearts overexpressing PL (203 ± 14 mmHg) to the same level as in uninfected (212 ± 12 mmHg) hearts. In addition, the isovolumic relaxation parameter τ in hearts infected with Ad.PL (12.8 ± 1.2 ms, $n = 6$) was decreased to levels similar to uninfected hearts (13.0 ± 1.1 ms, $n = 6$).

Pressure–Dimension Relationship. To further define ventricular function, pressure–dimension analysis was performed in a subset of animals. The relationship between developed pressure and ventricular dimension under different loading conditions allows accurate assessment of systolic and diastolic function. Fig. 6 shows a pressure–dimension relationship in an uninfected heart and a heart infected with Ad.PL at 2 days. The pressure–dimension loop is shifted to higher ventricular dimensions in the heart overexpressing PL. To alter loading conditions, we clamped the inferior vena cava in the open-chested animals, thereby reducing ventricular volume. This procedure enabled us to calculate the maximal end-systolic pressure–dimension relationship with a series of measurements made under varying preload conditions. The maximal slope of the end-systolic pressure–dimension relationship was lower in hearts overexpressing PL, indicating a diminished state of intrinsic myocardial contractility: 72 ± 21 mmHg/mm vs. 128 ± 24 mmHg/mm in Ad.PL-infected hearts ($n = 6$) compared with uninfected hearts ($n = 6$) [$P < 0.05$].

DISCUSSION

In this study we used a catheter-based technique to achieve global cardiac transduction with recombinant adenoviral vectors. Transgene expression was diffuse and relatively homogeneous throughout the myocardium. Importantly, transduction with adenoviral vectors carrying reporter genes did not significantly alter any of the physiological parameters. In contrast, cardiac overexpression of PL at 2- to 3-fold the endogenous level dramatically modulated global left ventricular function, recapitulating many of the abnormalities of human and experimental heart failure.

Cardiac Gene Transfer. Although prior studies have demonstrated the feasibility of cardiac gene transfer, the general

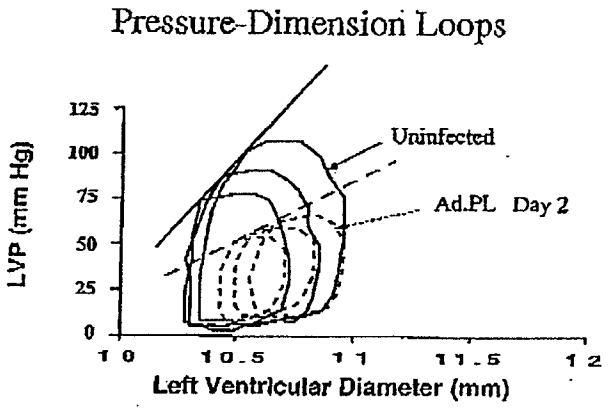


FIG. 6. Left ventricular pressure vs. left ventricular dimension detected by piezoelectric crystals in a control uninfected heart and in a heart infected with Ad.PL (5×10^9 pfu, day 2) under different loads obtained by clamping the inferior vena cava.

utility of these approaches has been limited by either very focal expression after *in vivo* transduction or the requirement of *ex vivo* infection for more diffuse transduction (12–15). Intracoronary catheter delivery of an adenovirus encoding β-gal achieved transduction of about 30% of the myocytes in the distribution of the coronary artery (12). Direct injection of adenovirus into the ventricular walls also induced significant expression of reporter constructs; however, the expression was focal and the injections within the myocardium cause needle damage (7, 8). More recently, Donahue *et al.* (15) reported effective gene transfer to the heart by using intracoronary perfusion in explanted hearts at physiological temperature. This group identified a number of parameters that influence the efficiency of adenoviral gene transfer. These included (*i*) the use of crystalloid solution as opposed to whole blood, (*ii*) coronary flow rate, (*iii*) exposure time, (*iv*) virus concentration, and (*v*) temperature. The success of the approach described here may reflect optimization of these parameters. The amount of virus used was 5×10^9 pfu diluted in 200 μl of solution and 150 μl from the left ventricular blood volume, resulting in a final concentration $\approx 1.5 \times 10^{10}$ pfu/ml. The concentration of the adenovirus was not diluted during the cross-clamping because blood return to the left atrium was minimized by the simultaneous clamping of the pulmonary artery. This concentration of virus is high compared with previous studies (7, 8, 15). In addition, the blood was diluted thereby decreasing the inhibitory effects of red blood cells on the efficiency of gene delivery. Even though we did not directly measure flow rates, cross-clamping increases perfusion pressure down the coronaries, allowing maximal opening of capillaries and optimizing the myocardial area of virus exposure. Furthermore, during cross-clamping the heart rate was decreased, thereby increasing diastolic time. During diastole, the left ventricular end-diastolic pressure was not significantly increased because blood return to the left ventricle was blocked by clamping of the pulmonary artery. Therefore perfusion of the virus could occur at relatively low downstream pressure and the endocardium could be efficiently infected. Because attachment of the adenovirus to cells is temperature dependent, delivering the virus *in vivo* allowed us to use the optimal temperature for adenoviral gene transfer, 37°C. Donahue *et al.* (15) have also shown that *in vivo* exposure of 10 s at a concentration of 10^7 pfu/ml results in ≈60% transduction studies. In the present study, where the delivery conditions have been optimized, the exposure time was 10 s at a much higher concentration of $\approx 10^{10}$ pfu/ml. In summary, many of the critical parameters previously characterized as important for effective gene transfer to myocytes, *in vitro* or *ex vivo*, are optimized during the described approach to cardiac gene transfer *in vivo*. Undoubtedly this contributes to its success and raises the possibility that *in vivo* global cardiac gene transfer will be feasible in other experimental or even clinical settings.

To assess the degree of gene expression with the delivery method described, we used two approaches. First, we visualized the whole heart by fluorescent microscopy after injection of Ad.EGFP. Second, we used histochemical staining to assess the microscopic distribution of transgene expression. Ad.EGFP infection produced grossly homogeneous expression. Of note, injection of the hearts with other viruses (carrying genes other than EGFP) exhibited no background fluorescence in this emission range, and direct injection into the ventricular wall of Ad.EGFP demonstrated intense green fluorescence at the site of injection but no background fluorescence in the surrounding tissue (Fig. 1). To further evaluate the microscopic distribution of transgene expression *in vivo*, the transduced hearts were examined with immunohistochemistry. We found that the distribution of β-gal staining was not uniformly diffuse in all cross-sections. Certain areas had diffuse staining, whereas in other sections a more patchy distribution of expression was observed. This staining may underestimate the extent of transgene expression both because the nuclear-localized β-gal construct used exhibits cytoplasmic activity only in highly expressing cells and because histochemical staining of β-gal is relatively insensitive. Lower levels of expression may not be detected by this method but may be sufficient to induce physiological changes, depending on the transgene expressed. Together our data with reporter constructs demonstrate highly effective gene transfer to adult rat hearts *in vivo* in a relatively homogeneous distribution.

Even though our delivery method was specifically targeted to the heart, we found expression of the reporter transgene in other tissues in the body, such as lung and liver but not aorta, by using histochemical staining. Other investigators have found extracardiac transgene expression following *in vivo* injection of adenovirus into the heart when using a nonspecific promoter (16, 17). The use of tissue-specific promoters may obviate this problem in the future.

PL Overexpression. Although such effective cardiac gene transfer with reporter constructs is encouraging, ultimately the utility of this approach depends on the ability to achieve functionally meaningful expression of biologically relevant transgenes. As a rigorous test of the system we chose to overexpress PL, an integral component of the SR thought to act only on the cell in which it is expressed. Expression of a secretory protein or a molecule with a significant bystander effect would be predicted to require less effective transduction to achieve a biological effect. Despite the stringent requirements of cell autonomous activity, PL had a dramatic effect on global left ventricular function.

PL is an integral protein of the SR of mammalian myocardium and regulates the Ca^{2+} ATPase that transports Ca^{2+} into the SR (3, 4). In the unphosphorylated state, PL inhibits the SERCA2a by reducing its affinity for Ca^{2+}. Phosphorylation of PL at either the Ser-16 site by cAMP-dependent protein kinase or the Thr-17 site by calmodulin-dependent mechanisms removes the inhibition to the SERCA2a. PL has been shown to be phosphorylated *in situ* and to contribute significantly to the positive inotropic response and the relaxant effects of β-agonism in the working heart (18). The expression of PL relative to SERCA2a has been shown to be altered in a number of diseased states (4, 19). Both human and experimental heart failure are associated with an increased ratio of PL/SERCA2a and are characterized by a prolonged calcium transient and impaired relaxation (20). In previous studies, we have shown that increasing levels of PL relative to SERCA2a in isolated cardiomyocytes prolonged the relaxation phase of the Ca^{2+}-transient, decreased Ca^{2+} release, and increased

resting Ca^{2+} (6). These abnormalities recapitulate in single cells many of the fundamental pathophysiologic abnormalities seen in heart failure. In the present study, overexpression of PL resulted in a decrease in systolic pressure, an increase in diastolic pressure, and a large increase in the time constant for isovolumic relaxation. Isovolumic relaxation, which is an index of active relaxation and reflects the removal of Ca^{2+} from the myofilaments into the SR, was significantly prolonged in the hearts overexpressing PL. This finding was also evident in the pressure–dimension relationship, which was characterized by a slowed early diastolic decline and opening of the mitral valve at higher filling pressures. The decrease in systolic pressure most likely reflects a decrease in SR Ca^{2+} loading. The SERCA2a is important both during relaxation by controlling the rate and amount of Ca^{2+} sequestered and during contraction by releasing the Ca^{2+} that is taken up by the SR. Overexpression of PL decreases SERCA2a activity, ultimately resulting in diminished systolic pressure and elevated diastolic pressure. In animals overexpressing PL, diastolic pressure was also elevated. PL has been shown to play a key role in modulating the response of agents that increase cAMP levels in cardiomyocytes (18, 21). Because cAMP-induced phosphorylation of PL reduces its inhibition of SERCA2a, we evaluated the effects of isoproterenol on the ventricular performance in hearts overexpressing PL. At maximal isoproterenol stimulation, the time course of isovolumic relaxation was decreased to levels similar to uninfected hearts and the LVSP was increased to levels similar to uninfected hearts. These results strongly suggest that the abnormalities observed in ventricular function in the hearts overexpressing PL are specifically the result of PL-mediated inhibition of SR ATPase. Pharmacological release of this inhibition restores ventricular function to the same level as uninfected hearts, reflecting the intrinsic SERCA2a activity that is the same in both cases.

A transgenic approach of overexpressing PL has been undertaken in mice. However, adenoviral transduction offers several advantages. In transgenic animals overexpressing PL, developmental adaptation to higher levels of PL occurs with up-regulation of other important excitation-contraction proteins such as the ryanodine receptor, thereby masking or diluting the effects of transgene overexpression (22). More recently, transgenic animals overexpressing another key protein involved in calcium regulation in myocytes, SERCA2a, have been shown to induce increases in the mRNA for PL and the sodium-calcium exchanger (23, 24). These compensatory alterations make it difficult to assess the specific effect of increasing PL on cardiac function. In our studies, overexpression of PL did not significantly alter protein expression of the ryanodine Ca^{2+}-releasing channels, SERCA2a, Na/Ca exchanger, or calsequestrin, which are all involved in intracellular calcium handling. However, adenoviruses have significant disadvantages that include the transient nature of overexpression of the desired gene and the immune/inflammatory response they produce and which was also present in our infected hearts. These shortcomings of the first generation adenoviruses limit their use in animal models over prolonged periods of time.

Although we noted extracardiac transgene expression, it is unlikely to account for the phenotype we observed. Although PL may regulate the SR Ca^{2+} pump in aortic smooth muscle cells (25), we found no histochemical evidence of β-gal expression in aortae. There is no known functional roles for these pathways in liver or lung, where we did detect extracardiac transgene expression. Moreover, we examined indices of systolic and diastolic ventricular performance that are independent of load (or changes in aortic compliance) and reflect intrinsic cardiac function. Our physiological data demonstrate the feasibility of achieving important functional cardiac effects through *in vivo* somatic gene transfer of a cell autonomous protein. Moreover, the abnormalities developed after PL overexpression *in vivo* reflect the abnormalities seen in experimental and human heart failure. Therefore, somatic gene transfer with Ad.PL can create both *in vitro* (6) and *in vivo* models of heart failure that should facilitate studies of pathophysiology and investigation of potential therapeutic interventions.

Conclusion. In conclusion, the present study demonstrates highly effective gene transfer to rat heart *in vivo*. By using this technique, we overexpressed PL, thereby recapitulating many of the abnormalities observed in heart failure. Together these studies open the prospect of using somatic gene transfer to modulate overall cardiac function *in vivo* for either experimental or therapeutic applications.

This work was supported in part by grants from the National Institutes of Health: HL 50361 and HL 57623 (R.J.H.); HL 54202, HL 59521, and AI 40970 (A.R.); and HL 49574 and HL 52349 (J.K.G.), and by donations to the Katherine Catani Memorial Fund (A.R.).

1. Barry, W. H. & Bridge, J. H. B. (1993) *Circulation* 87, 1806–1815.
2. Gwathmey, J. K., Bentivegna, L. A., Ransil, B. J., Grossman, W. & Morgan, J. P. (1993) *Cardiovasc. Res.* 27, 199–203.
3. Koss, K. L. & Kranias, E. G. (1996) *Circulation Res.* 79, 1059–1063.
4. Arai, M., Matsui, H. & Periasamy, M. (1994) *Circ. Res.* 74, 555–564.
5. Hajjar, R. J., Kang, J. X., Gwathmey, J. K. & Rosenzweig, A. (1997) *Circulation* 95, 423–429.
6. Hajjar, R. J., Schmidt, U., Kang, J. X., Matsui, T. & Rosenzweig, A. (1997) *Circ. Res.* 81, 145–153.
7. Guzman, R. J., Lemarchand, P., Crystal, R. G., Epstein, S. E. & Finkel, T. (1993) *Circ. Res.* 73, 1202–1207.
8. French, B. A., Mazur, W., Geske, R. S. & Bolli, R. (1994) *Circulation* 90, 2414–2424.
9. Dong, G., Schulick, A., DeYoung, M. B. & Dichek, D. A. (1996) *J. Biol. Chem.* 271, 29969–29977.
10. Harigaya, S. & Schwartz, A. (1969) *Circulation Res.* 25, 781–794.
11. Bradford, M. (1976) *Anal. Biochem* 72, 248–260.
12. Barr, E., Carroll, J., Kalynych, A. M., Tripathy, S. K., Kozarsky, K., Wilson, J. M. & Leiden, J. M. (1994) *Gene Ther.* 1, 51–58.
13. Kirshenbaum, L. A., MacLennan, W. R., Mazur, W., French, B. A. & Schneider, M. D. (1993) *J. Clin. Invest.* 92, 381–387.
14. Kass-Eisler, A., Falck-Pedersen, E., Alvira, M., Rivera, J., Buttrick, P. M., Wittenberg, B. A., Cipriani, L. & Leinwand, L. A. (1993) *Proc. Natl. Acad. Sci. USA* 90, 11498–11502.
15. Donahue, J. K., Kikkawa, K., Johns, D. C., Marban, E. & J., L. (1997) *Proc. Natl. Acad. Sci. USA* 94, 4664–4668.
16. Kass-Eisler, A., Falck-Pedersen, E., Alvira, M., Rivera, J., Buttrick, P. M., Wittenberg, B. A., Cipriani, L. & Leinwand, L. A. (1993) *Proc. Natl. Acad. Sci. USA* 90, 11498–11502.
17. Kass-Eisler, A., Falck-Pedersen, E., Elfenbein, D. H., Alvira, M., Buttrick, P. M. & Leinwand, L. A. (1994) *Gene Ther.* 1, 395–402.
18. Tan, W., Grupp, I. L., Harrer, J., Ponniah, S., Grupp, G., Duffy, J. J., Doetschman, T. & Kranias, E. G. (1994) *Circulation Res.* 75, 401–409.
19. O'Brien, P. J. & Gwathmey, J. K. (1995) *Cardiovasc. Res.* 30, 394–404.
20. Gwathmey, J. K. & Morgan, J. P. (1993) *Pflügers Arch.* 422, 599–608.
21. Karczewski, P., Bartel, S. & Krause, E.-G. (1990) *Biochem. J.* 266, 115–122.
22. Chu, G., Luo, W., Slack, J. P., Tilgmann, C., Sweet, W. E., *et al.* (1996) *Circ. Res.* 79, 1064–1076.
23. He, H., Giordano, F. J., Hilal-Dandan, R., Choi, D. J., Rockman, H. A., *et al.* (1997) *J. Clin. Invest.* 100, 380–389.
24. Giordano, F. J., He, H., McDonough, P., Meyer, M., Sayen, M. R. & Dillmann, W. H. (1997) *Circulation* 96, 400–403.
25. Lalli, J., Harrer, J. M., Luo, W., Kranias, E. G. & Paul, R. J. (1997) *Circ. Res.* 80, 506–513.

EXHIBIT T

cause lethal graft-versus-host disease across minor histocompatibility barriers in mice. J Exp Med 1987; 165 (6): 1552.
62. Korngold R, Leighton C, Manser T. Graft-versus-myeloid leukemia responses following syngeneic and allogeneic bone marrow transplantation. Transplantation 1994; 58 (3): 278.
63. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood 1997; 90 (8): 3204.
64. van den Brink MRM, Moore E, Ferrara JLM, Burakoff SJ. Graft-versus-host disease-associated thymic damage results in the appearance of T cell clones with anti-host reactivity. Transplantation 2000; 69: 446.
65. Hollander GA, Widmer B, Burakoff SJ. Loss of normal thymic repertoire selection and persistence of autoreactive T cells in graft vs host disease. J Immunol 1994; 152 (4): 1609.
56. Hakim FT, Mackall CL. The immune system: effector and target of graft-versus-host disease. In: Ferrara JLM, Deeg HJ, Burakoff SJ, eds. Graft-vs.-host disease. New York: Marcel Dekker, 1997: 257.
57. Piguet PF, Grau GE, Allet B, Vassalli P. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. J Exp Med 1987; 166 (5): 1280.
58. Herve P, Flesch M, Tiberghien P, et al. Phase I-II trial of a monoclonal anti-tumor necrosis factor alpha antibody for the treatment of refractory severe acute graft-versus-host disease. Blood 1992; 79 (12): 3362.

Received 17 August 1999.
Accepted 2 February 2000.

0041-1337/00/7001-191/0
TRANSPLANTATION

Vol. 70, 191–198, No. 1, July 15, 2000
Printed in U.S.A.

IMMUNE RESPONSE AFTER ADENOVIRAL GENE TRANSFER IN SYNGENEIC HEART TRANSPLANTS: EFFECTS OF ANTI-CD4 MONOCLONAL ANTIBODY THERAPY[1]

GRIT SCHRÖDER,[2,7] KIRSTEN RISCH,[2] HORST NIZZE,[3] JAY KOLLS,[5] PETRA REINKE,[6] JOSEF BROCK,[2] MANFRED LEHMANN,[2] HANS-DIETER VOLK,[4] AND THOMAS RITTER[4]

Institute of Medical Biochemistry and Molecular Biology and Institute of Pathology, University Rostock; Institute of Medical Immunology and Department of Nephrology and Internal Intensive Care, Charité, Humboldt-University Berlin, Germany; LSU Section of Pulmonary & Critical Care, MEB, New Orleans, LA 70112-1393

Background. E1-deleted adenoviral vectors are frequently used for in vivo gene therapy. However, gene expression after adenovirus- (ad) mediated gene transfer is known to be transient due to the generation of an immune response against virus-infected cells. In this study, we asked whether an anti-CD4 mAb (RIB 5/2) treatment may improve the gene transfer into rat cardiac grafts.

Methods. We injected recombinant ad-constructs encoding for *Escherichia coli* β-gal into syngeneic rat heart transplants via the proximal aorta. One-half of the recipients of genetically modified grafts received the anti-CD4 mAb RIB 5/2, whereas the other half received no monoclonal antibody treatment. Genetically unmodified isografts without any treatment of the recipients were used as additional controls. At different time points hearts were harvested and analyzed for reporter gene expression, intragraft cellular infiltration, and cytokine gene expression (quantitative "real time" reverse transcriptase polymerase chain reaction). Serum samples were analyzed for anti-ad-Ig using enzyme-linked-immunosorbent-assay.

Results. In control animals the β-gal reporter gene expression slowly increased until day 7 and then declined. The immunohistological and reverse transcriptase polymerase chain reaction intragraft analyses revealed a strong inflammatory response (cellular infiltration, cytokine expression) in ad-transfected grafts that may explain the delayed expression and fast down-regulation of the transgene. Treatment with RIB 5/2 mAb resulted in a faster and prolonged reporter gene expression, reduced graft infiltration, reduced anti-ad-Ig titers and less interferon-γ up-regulation.

Conclusions. Our results indicate that modulation of the anti-ad immune response using a nondepleting anti-CD4 mAb may increase the efficiency of ad-vectors for gene therapy in the transplant setting.

The possibility to transfer genes into organ grafts has been an attractive approach to modulate posttransplant events. Potential therapeutic applications include reduction of the ischemia/reperfusion injury, prevention of acute and chronic rejection, and induction of tolerance. However, effective gene therapy requires an efficient system of gene delivery and transgene expression within the graft. Recombinant adeno-

[1] Supported by the Deutsche Forschungsgemeinschaft (Vo 489/6–

[2] Institute of Medical Biochemistry and Molecular Biology.
[3] Institute of Pathology, University Rostock.
[4] Institute of Medical Immunology.
[5] LSU Section of Pulmonary & Critical Care, MEB.
[6] Department of Nephrology and Internal Intensive Care.
[7] Address correspondence to: Grit Schröder, Institute of Medical Biochemistry and Molecular Biology, Schillingallee 70, 18057 Rostock, Germany.

viruses have proved to be reliable vectors for successful gene transfer into different tissues before transplantation (1–3).

Compared with other gene transfer systems, recombinant adenoviruses have several advantages. Adenovirus- (ad) vectors are able to transduce nonreplicative cells. Moreover, E1-deleted ad-vectors are replication-deficient and can be grown to high titers (4). Adenoviruses are usually not incorporated into the host cell genome, thus avoiding the risk of insertional mutagenesis. Disadvantages of ad-mediated gene transfer include the transient transgene expression and the immunogenicity of transduced cells. The resulting immune response is composed of both the generation of CTL against virally transduced cells and the development of a neutralizing antibody response (5, 6).

In the transplant situation the gene transfer approach is even more complicated. The ischemia/reperfusion injury induces a local inflammatory response that further increases the immunogenicity of the ad-transfected cells in the graft (7). Moreover, proinflammatory cytokines, in particular interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), may down-regulate the CMV-IE promoter that is commonly used for the control of the transgene (8, 9). In preliminary studies we observed a delayed reporter transgene expression in transplants in comparison to nontransplanted organs, even in the syngeneic models. As the immunomodulatory activity of the transgene is especially required immediately after transplantation, a delayed transgene expression reduces its therapeutic efficacy. However, a strong antiviral immune response also supports alloreactivity. Therefore, the ad-mediated gene transfer approach is limited in the transplant setting.

Several strategies have been developed in nontransplantation models to overcome the immune response to ad-vectors including the blockade of costimulatory signals to T cells by CTLA4-Ig, and the application of depleting or non-depleting anti-CD4 mAb (10–12). We wondered whether a nondepleting anti-CD4 mAb used at a low dose (10×1 mg/kg/day) which is not able to induce long-term allograft survival, may improve the efficacy of ad-mediated gene transfer into cardiac grafts. We found that low dose anti-CD4 mAb treatment improved the early and late transgene expression, reduced the graft infiltration and the anti-ad-antibody response and attenuated the intragraft up-regulation of IFNγ. This approach may expand putative applications of ad-mediated gene transfer in allotransplantation models.

MATERIALS AND METHODS

Animals. Male inbred rats of the Lewis (RT1^{l}) and the DA (RT1^{av1}) genetic background aged 8–12 weeks, were purchased from Mollegaard Breeding Centre Ltd., Ry, Denmark, and were used for syngeneic and allogeneic heart transplantation.

Adenoviral vectors. For gene transfer studies recombinant E1-deleted, first-generation replication-incompetent ad type 5 vector encoding for *Escherichia coli* β-gal under the control of the human CMV-IE promoter (ad5CMVβgal) was used. Viruses were propagated, purified, and titered on 293 cells as described previously (4). Virus concentration was determined by OD and by plaque assay.

mAb treatment. RIB 5/2 (a nondepleting mouse anti-rat CD4 mAb) was purified from ascites by protein A affinity chromatography and the concentration was determined by enzyme-linked-immunosorbent-assay (ELISA) using IgG2a standards (Sigma, Deisenhofen, Germany) (13, 14).

Animals grafted with ad-infected syngeneic hearts on day 0 (see below) received injections i. p. with 4 mg/kg of RIB 5/2 daily from day −1 to day 3 and thereafter twice per week for 2 weeks. Control animals received ad-infected grafts without RIB 5/2 treatment. Another control group received nontransfected grafts and was also not treated with RIB 5/2.

Heterotopic heart transplantation and gene transfer. Syngeneic and allogeneic heterotopic heart transplantation was performed using standard microsurgical technique with end-to-side anastomosis of aorta abdominalis (recipient) to aorta (donor) and vena cava (recipient) to arteria pulmonalis (donor) (15).

For ex vivo gene transfer hearts prepared from the donors were rinsed with protective solution (Custodiol, Dr. Franz Köhler Chemie GmbH, Alsbach-Hähnlein, Germany). After injection of 5×10^{8} plaque forming units (pfu) of ad5CMVβgal into the donor aorta from animals of the first and second group, heterotopic heart transplantation was performed in the abdominal localization. The cold ischemic time was about 60 min. Graft survival was checked twice a week until the end of the observation period.

Syngeneic recipients were killed at days 3, 7, 14, 21, 28, and 56 and allogeneic recipients at days 3 and 5, respectively. The heart transplants were removed and rinsed with phosphate-buffered saline (PBS). The basal and apical section (about 3 mm each) were separately embedded in OCT compound (Life Sciences International, Europe, LTD. Astmoor, England) and snap-frozen in liquid nitrogen for X-gal staining and immunohistochemistry. The middle part of the heart transplant was analyzed for *E. coli* β-gal protein expression by ONPG assay and for cytokine mRNA expression by reverse transcriptase polymerase chain reaction (RT-PCR) (see below).

In situ X-gal staining. Cryostat sections were stained for X-gal as described before (12). Briefly, six 10-μm thick sections of the basal and the apical part were cut at 100-μm intervals. Specimens were fixed in acetone for 5 min at 4°C and then rinsed twice with PBS. After staining in a solution of 1.5 mM $K_4Fe(CN)_6$, 1.5 mM $K_3Fe(CN)_6$, 0.2 mM $MgCl_2$, 400 μg/ml X-gal in PBS for 24 hR at 37°C sections were rinsed in water and counterstained with hemalaune. Blue-stained cells indicated the presence of β-gal protein expression. For quantitative analysis, the total number of positive stained cells was counted for each section under light microscope and normalized for the area. The area of each section was measured using a Nikon SMZ-10A microscope with a CCD color vision camera module (Doupisha, Sony). Each image was analyzed using Biosecan Optimas (Biosecan, Inc., Paardeweide, Netherlands) software.

o-nitrophenol-β-D-galactoside analysis (ONPG assay). For quantitative reporter gene expression studies hearts were analyzed for expression of β-gal using an ONPG assay. Specimens were homogenized in 0.1 M potassium phosphate buffer with 0.2% Triton X-100 (pH 7.8) and 0.2 mM protease inhibitor, 4-[2-aminoethyl]-benzensulfonylfluoridhydrochloride (Boehringer Mannheim, Mannheim, Germany). Cell lysates were then centrifuged at 16.000 × *g* for 10 min at 4°C. For heat inactivation of endogenous β-gal activity supernatants were incubated for 60 min at 48°C and then centrifuged at 16.000 × *g* for 10 min at 4°C. Enzymatic activity of the supernatants was measured in a reaction volume of 1 ml consisting of 30 μl cell lysate, 770 μl PM-2 buffer (23 mM NaH_2PO_4, 77 mM Na_2HPO_4, 0.1 mM $MgCl_2$, 2 mM $MgSO_4$, 40 mM β-mercaptoethanol, 0.1% Triton X-100, pH 7) and 200 μl ONPG-solution (4 mg/ml ONPG in PM-2-buffer). After 30 min at 37°C, the reaction was stopped by adding 500 μl 1 M Na_2CO_3. The optical density of each sample was then read at 420 nm to determine activity as previously described (16). Activity was normalized for protein concentration, which was determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Immunohistochemistry. An alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used to analyze cellular graft infiltration. The 6-μm thick cryostat sections of the apical part were fixed in acetone for 10 min at room temperature. To block nonspecific antibody binding, slides were incubated in 20% rabbit serum/EACS buffer (0.1% NaN_3, 1% fetal calf serum in PBS) for 20 min. Sections were incubated with mouse antibodies against rat T cell receptor (α

(R73), CD8-β chain (341), p55 chain of interleukin- (IL) 2R (ART18), and macrophages, monocytes, dendritic cells (ED1) for 30 min. Hybridoma cells were kindly provided by T. Hünig/Würzburg and T. Diamantstein/Berlin, respectively. Slides were washed three times with buffer, then incubated with the second antibody (rabbit anti-mouse immunoglobulin, DAKO, Hamburg, Germany) at a 1:50 dilution (containing 10% rat serum) for 30 min. After extensive washing, sections were incubated with APAAP complex (Quartett, Berlin, Germany) at a dilution of 1:50 (containing 10% rat serum) for 30 min. The APAAP reaction was visualized with New-Fuchsin and naphtholphosphate. Slides were counterstained with hemalaunne, dried and embedded in glycerole gelatine. The tissue sections were evaluated under the light microscope (magnification 400) by counting positive cells of an area of 0.8 mm^2.

Adenovirus ELISA. Ad antigen (ad in PBS, sonicated for 5 min) was resuspended in carbonate buffer (34 mM Na_2CO_3, 16 mM $NaHCO_3$, pH 9.5) to a final concentration of 40 ng/100 μl. ELISA plates were coated with ad-antigen by pipetting 100 μl/well and incubated overnight. Plates were washed (wash buffer: PBS/0.05% Tween 20) and then blocked by adding 200 μl/well of blocking buffer (PBS, 0.5% bovine serum albumin (BSA), 0.05% sodiumazide, and 2% skim milk) for 1 hr at room temperature. After washing serial dilutions of the serum samples (in PBS/1% BSA) were added to the wells and incubated for 1 hr at room temperature. Then the plates were incubated with 100 μl/well of sheep anti-rat Ig POD Fab fragments (Boehringer Mannheim, dilution 1:1000 in PBS/1% BSA) for 1 hr. After an incubation for 15 min at room temperature with 100 μl/well OPD-solution (2 mM 1,2-phenylendiamine, 49 mM citric acid · H_2O, 100 mM Na_2HPO_4 · 2 H_2O, 0.03% H_2O_2) the reaction was stopped with 50 μl/well 0.1 M H_2SO_4. Plates were then read at 492 nm on a plate reader (anthos reader 2001, Salzburg, Austria).

Quantitative RT-PCR. IFNγ and TNFα gene transcription in the syngeneic rat hearts were analyzed using quantitative "real-time" RT-PCR using the TaqMan system (Perkin-Elmer Applied Biosystems, Moersheim, Germany) (*17*). Total RNA was prepared from biopsies of each graft using RNaid-kit (Dianova, Hamburg, Germany) and reversely transcribed into cDNA by the moloney murine leukemia virus reverse transcriptase (GibcoBRL, Heidelberg, Germany). The cDNA was then analyzed for cytokine gene expression with the "real time" PCR. Primers and oligonucleotide hybridization probes were designed using the software purchased by the TaqMan supplier (Perkin-Elmer Applied Biosystem). All other PCR reagents were obtained from Boehringer Mannheim. Tubes of amplification reactions (25 μl) contained 1 μl of the cDNA sample, 10x TaqMan buffer A (2.5 μl), 800 μM dNTPs (dATP, dUTP, dCTP, dGTP), 6 mM $MgCl_2$, 0.625 U AmpliTaq gold polymerase, 0.25 U AmpErase uracil N-glycosylase (UNG), 0.2 pM probe, 300 nM sense primer, 900 nM antisense primer. MicroAmp optical tubes and MicroAmp optical caps (Perkin Elmer Applied Biosystems) were used. The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Reactions were performed in the Model 7700 Sequence Detector (Perkin Elmer Applied Biosystems). The cycle number at which the reporter fluorescence crosses the threshold (C_T value) was used for quantitative measurement. Values are given as delta C_T levels of IFNγ or TNFα versus GAPDH (housekeeping gene) expression.

Statistical analysis. Values are reported as mean±SD. Group comparisons were performed using the parameter-free *U* test. Differences were considered significant at $P<0.05$.

RESULTS

Kinetics of transgene expression in the heart transplants after adenoviral gene transfer. Successful gene transfer and expression of β-gal was documented in all animals using X-gal staining. Negative controls (nontransfected heart transplants and recipient's hearts) showed no positive staining (Fig. 1). To examine the transgene expression in transplanted organs, β-gal expressing cells were counted in syngeneic heart grafts at day 3, 7, 14, 21, 28, and 56 after ad-gene transfer (Fig. 2A). Whereas in nontransplanted organs the ad-transfected cells express the reporter gene within 2 to 3 days (*4, 12, 18*), in our experiments heart transplants reached peak levels of β-gal activity not before day 7. Thereafter, the expression decreased and only few positive cells could be detected at day 28 and 56 after gene transfer. The measurement of the total β-gal enzymatic activity of the transfected cardiac tissue using the ONPG-assay revealed similar results (Fig. 2B) suggesting a delayed expression and fast down-regulation of the reporter gene in cardiac grafts. Moreover, we analyzed the reporter gene expression in the presence of allogeneic grafts in the model DA to LEW. At days 3 and 5 successful gene transfer was documented in all animals and the transgene expression was not significantly lower than in syngeneic hearts (data not shown). These results suggest that the early transgene expression is not limited in the allotransplantation model.

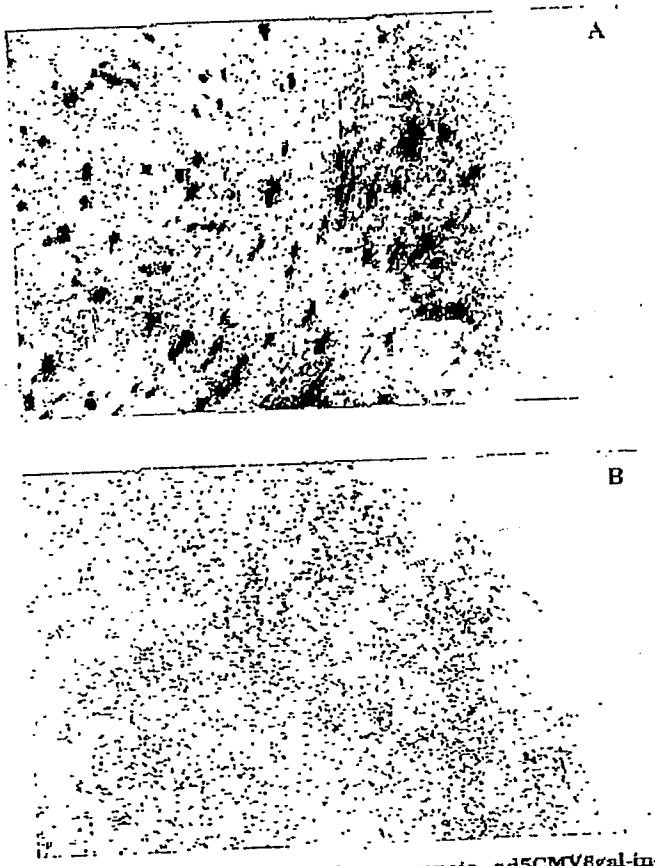


FIGURE 1. β-Gal expression in syngeneic ad5CMVβgal-infected rat hearts. A, X-gal staining of syngeneic rat heart 7 days after ad5CMVβgal gene transfer (original magnification ×12,8). B, Recipient's own heart demonstrated no positive staining.

Cellular infiltration of the syngeneic heart transplants after adenovirus-mediated gene transfer. We hypothesized that the ischemia/reperfusion injury mediated inflammation contributes to the delayed expression and fast elimination of the reporter gene in cardiac grafts. As shown for alloreactivity,

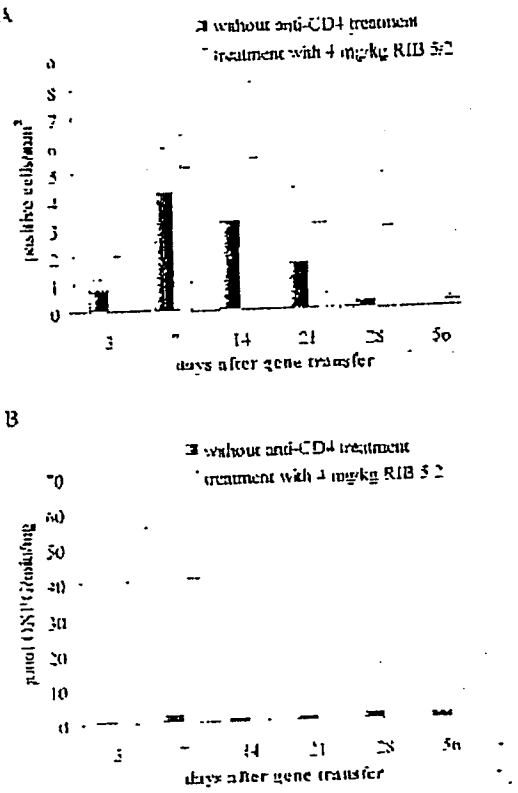


FIGURE 2. Kinetics of transgene expression in syngeneic heart transplants after adenoviral gene transfer. A, X-gal expressing cells: Ad-treated heart transplants were removed at different time points and the β-gal expression was analyzed by X-gal staining (n=5–7/group). The number of X-gal$^+$ cells was significantly higher in anti-CD4 mAb-treated (white bars) versus untreated control rats (black bars) at days 28 and 56 ($P<0.05$). B. Total tissue enzymatic activity: Ad-treated heart transplants were removed at different time points and the total tissue β-gal expression was analyzed by the ONPG assay (n=5/each group). The enzymatic activity was significantly higher in anti-CD4 mAb treated (white bars) versus control rats (black bars) at days 3, 7, 14, 21, and 28 ($P<0.05$).

the unspecific inflammation following ischemia/reperfusion injury may increase the specific immunogenicity of transfected cells in the graft. To study the inflammatory response, the cellular infiltration of transfected syngeneic grafts by cells expressing the α/β T cell receptor, CD8 β chain, IL-2 receptor or a membrane marker expressed on macrophages, monocytes, and dendritic cells (detected by ED-1) was analyzed in comparison to untreated syngeneic heart transplants (Fig. 3, A-D).

The ischemia/reperfusion injury resulted in increasing cellular infiltration until day 14 after transplantation (Fig. 3, A-D, see *Nontransfected grafts*). The ad-mediated transfection of the grafts with the reporter gene β-gal further increased the cellular infiltration three- to 7-fold (Fig. 3, A-D, see *No mAb treatment*). Interestingly, the inflammatory response showed a biphasic course in ad-transfected grafts suggesting an early inflammation (day 3) triggered by unspecific events and a later (after day 14) cellular infiltration that may result from the ad-specific immune response.

Effect of RIB 5/2 mAb treatment on transgene expression after adenoviral gene transfer. Next, we wondered whether the anti-CD4 mAb RIB 5/2 may deteriorate the specific anti-ad immune response and prolong the expression of the transgene within the graft. The graft recipients received 10 times 4 mg/kg of the mAb, a suboptimal dose that is not able to induce long-term allograft survival (*11*). We did not use the high-dose allograft tolerance-inducing dose (10×20 mg/kg) as tolerance induction to the adenovirus may be dangerous for a clinical setting. This low-dose anti-CD4 mAb protocol significantly prolonged the reporter gene expression in transplanted syngeneic hearts in comparison to control hearts without anti-CD4 treatment (Fig. 2, A and B). At days 28 and 56 after transplantation, the number of X-gal positive cells in anti-CD4 mAb treated rats was 12- and 11-fold higher, respectively, in comparison with the mAb untreated rats (Fig. 2A). Moreover, the early expression of the transgene within the first week was also significantly improved under the umbrella of RIB 5/2 mAb treatment, which could be detected measuring the total enzyme activity by the ONPG-assay (Fig. 2B).

Effect of RIB 5/2 mAb treatment on cellular graft infiltration after adenoviral gene transfer. The improved gene transfer into syngeneic cardiac grafts by anti-CD4 mAb treatment was associated with a down-regulation of both the early and late inflammatory response. RIB 5/2 mAb treatment prevented the ad-mediated up-regulation of the intragraft inflammatory response. Moreover, the number of infiltrating T cells was even lower than in the untreated syngeneic non-transfected control hearts (Fig. 3, A and B), although the infiltration by IL-2R$^+$ T cells was not completely prevented suggesting some residual anti-ad specific immune response (Fig. 3C). The late infiltration by ED-1$^+$ macrophages was also reduced, whereas the early infiltration by these cells was also not completely prevented by anti-CD4 mAb treatment (Fig. 3D).

Effect of RIB 5/2 treatment on antiadenoviral antibody production. To examine the humoral immune response to the ad-vectors we have quantified the formation of anti-ad antibodies in anti-CD4 mAb-treated or mAb-untreated animals receiving syngeneic ad-infected grafts by ELISA technology. We observed higher titers of anti-ad antibodies in comparison to background levels from animals without gene transfer from day 21 to day 47 after gene transfer. Anti-CD4 mAb-treated animals showed a strongly reduced and delayed production of anti-ad antibodies compared with animals without mAb treatment suggesting a reduced humoral immune response to the ad-vectors (Fig. 4).

Effect of RIB 5/2 treatment on proinflammatory cytokine gene transcription after adenoviral gene transfer. It is well established that proinflammatory cytokines, particularly IFNγ and TNFα, may down-regulate the transgene expression by inactivation of the commonly used CMV-IE-promoter. We observed a strong up-regulation of IFNγ and TNFα mRNA expression in the transfected hearts within the first 2 days. The early intragraft IFNγ mRNA expression was dramatically reduced in anti-CD4 mAb-treated graft recipients (Fig. 5A). Levels of IFNγ mRNA in anti-CD4 mAb-untreated β-gal-infected syngeneic grafts were 3- to 4-fold higher compared to syngeneic otherwise untreated controls (data, not

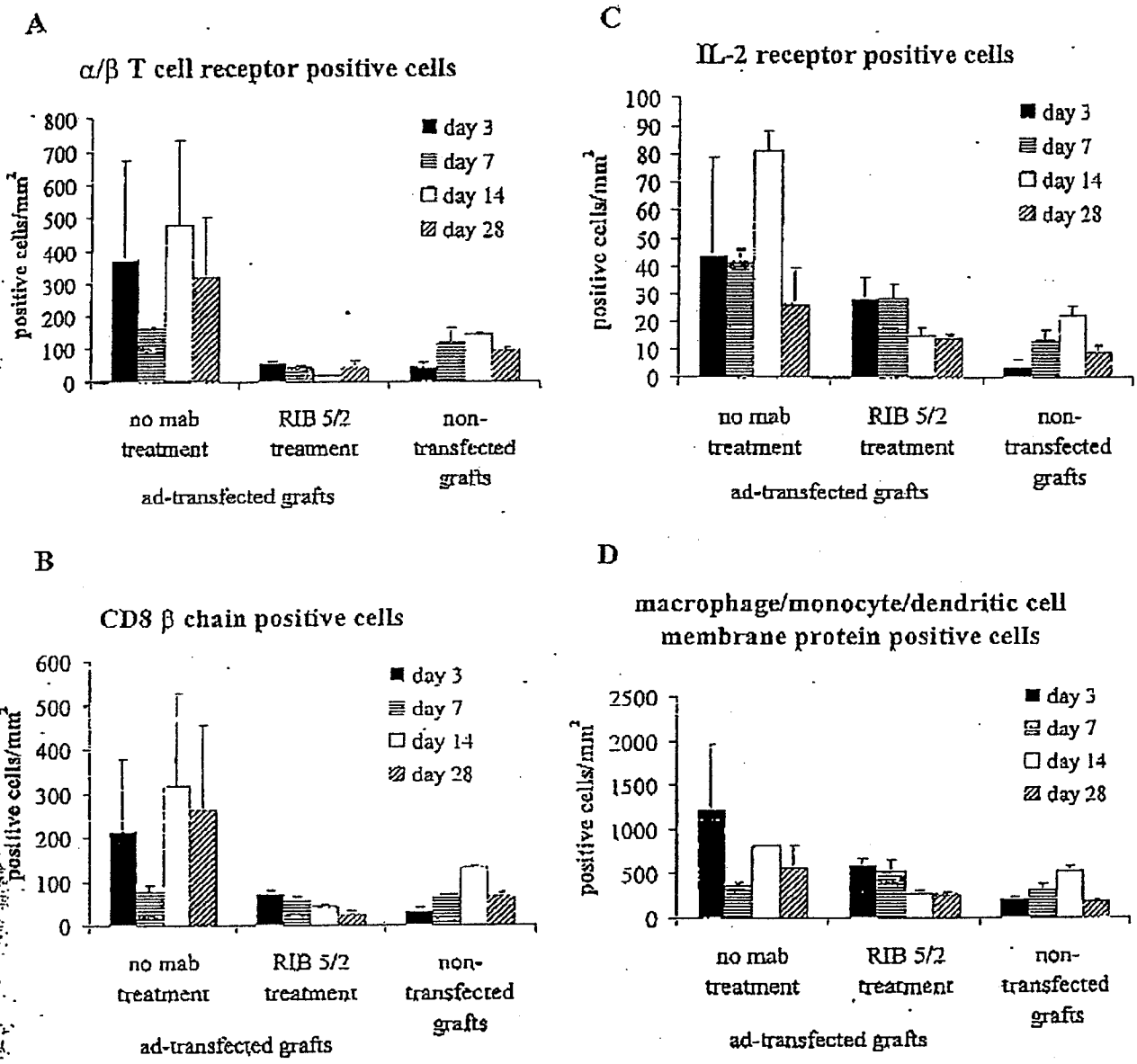


FIGURE 3. Cellular infiltration of the syngeneic heart transplants after adenovirus-mediated gene transfer. Ad-treated heart transplants with or without anti-CD4 therapy and untransfected control heart transplants were removed at different time points and analyzed for infiltration with (A) α/β T cell receptor positive cells, (B) CD8 β chain positive cells, (C) IL-2 receptor positive cells, (D) ED-1⁺ cells (marker for macrophages, monocytes, and dendritic cells) (n=4–5/each group). Anti-CD4 mAb treatment significantly attenuated the graft infiltration in comparison to untreated ad-transfected grafts at day 3, 14, and 28 ($P<0.05$).

shown). This suggests that the increase in levels of IFNγ at day 3 after gene transfer in anti-CD4 mAb-untreated animals is mainly due to the presence of adenovirus. In contrast, the TNFα response was not significantly influenced (Fig. 5B) despite the reduced cellular infiltration (Fig. 3, A-D).

DISCUSSION

Recombinant ad-vectors are an useful tool for efficient gene transfer into a variety of organ transplants (*18–22*). However, potential applications of this vector system are limited by transient transgene expression after gene transfer. In nontransplanted organs the maximum level of reporter gene expression is reached within the first 24 to 48 hr after ad-mediated gene transfer and remains stable for 3 to 4 weeks (*4, 12*). In this study we observed a delayed expression and fast elimination of the transgene in a syngeneic cardiac transplantation model suggesting that the unspecific inflammatory response after ischemia/reperfusion injury of grafts further deteriorates the transgene expression in the transplant setting.

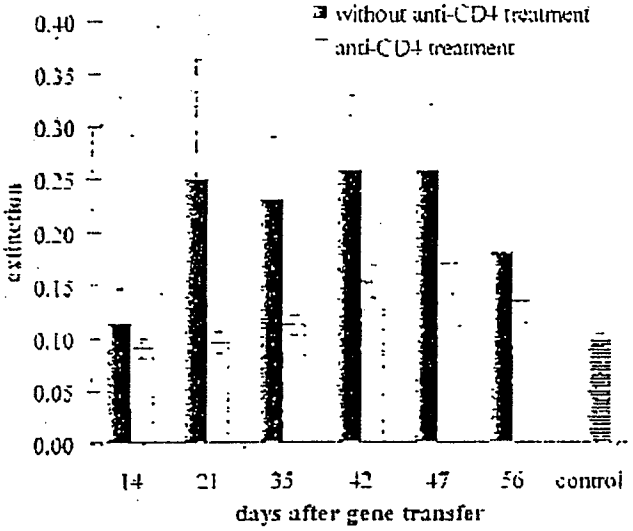
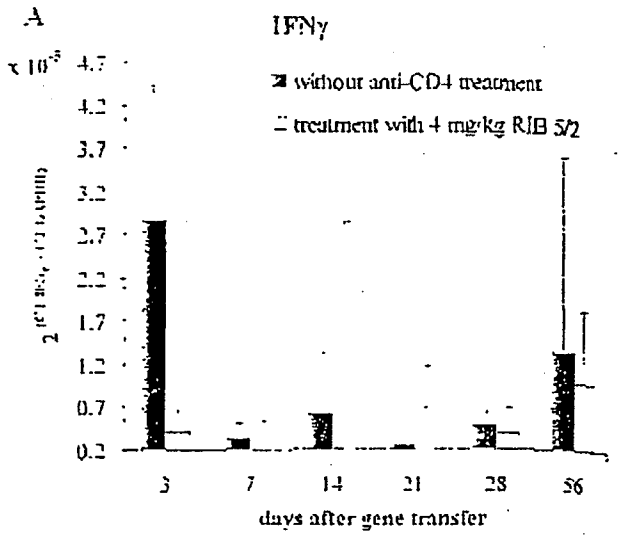


FIGURE 4. Effect of RIB 5/2 treatment on anti-adenoviral antibody production. Serum samples of animals receiving mAb- and mAb-untreated animals were analyzed using an anti-ad-Ig detecting ELISA. Values are given as extinction at the 1:128 dilution of the serum samples. Background levels were determined measuring control serum of ad5CMVβgal-untreated animals (striped bar). Anti-CD4 mAb treatment (white bars) significantly reduces the amount of anti-ad antibodies compared with mAb-untreated animals (black bars) (n=4–5/each group).



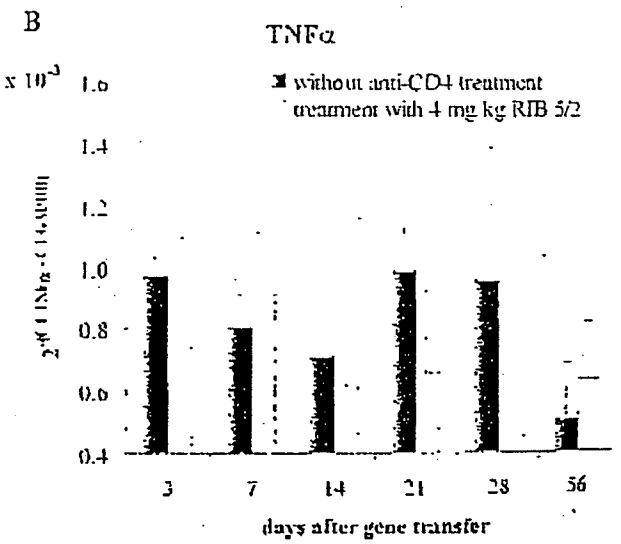
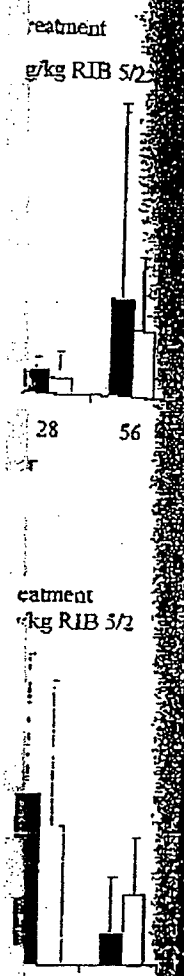


FIGURE 5. Effect of RIB 5/2 treatment on proinflammatory cytokine gene transcription after adenoviral gene transfer. Ad-transfected heart transplants from anti-CD4-treated rats and nontreated rats were removed at different time points and analyzed for (A) IFNγ mRNA expression and (B) TNFα mRNA expression by quantitative "real time" TaqMan-RT-PCR. C_T Threshold-cycle (n=4/each group). Values are given as 5 C_T levels of IFNγ or TNFα versus GAPDH (housekeeping gene) expression. Anti-CD4 mAb treatment significantly ($P<0.05$) reduced IFNγ gene expression at day 3.

The low reporter gene expression within the first days after transplantation was associated with a huge early intragraft accumulation of both macrophages and T cells in ad-transfected grafts in comparison to untreated grafts that was accompanied by a strong up-regulation of IFNγ. After day 3 cellular infiltration and intragraft IFNγ expression spontaneously regressed. As the E1-deleted ad is replication deficient, the rising frequency of X-gal positive cells between day 3 and 7 does not reflect increasing numbers of transfected cells but rather indicates a strong down-regulation of the commonly used CMV-promoter during the first days as result of the early inflammatory response. It has been shown that cytokines may down-regulate the CMV-IE promoter activity (*24, 8*). The action of TNFα (up-regulation, down-regulation, or no effect) on the CMV-IE promoter activity is dependent on the target cells (*8, 25*), whereas IFNγ seems to inhibit the promoter in all cell lineages (*8, 9*).

With the reduction of both cellular infiltration and intragraft IFNγ expression between day 3 and 7, the transgene expression increases. TNFα expression did not significantly change during the observation time suggesting that TNFα is less involved in promoter down-regulation in our in vivo model.

However, after day 14 a second wave of graft infiltration appeared that was associated with a decreasing reporter gene expression (both frequency of X-gal$^+$ cells and total enzyme activity went down). These late events probably reflect the specific immune response to ad-encoded proteins that results in the elimination of the transduced cells. In the transplant setting the early ischemia/reperfusion injury-mediated inflammation activates intragraft antigen-presenting cells, induces the release of chemotactic factors, and up regulates endothelial adhesion molecules; thus increasing the immunogenicity of the ad-infected cells and supporting the development of the specific anti-ad immune response. Even though it has been considered that the major cause of the transient transgene expression after adenoviral gene transfer is the development of an adaptive immune response to adenoviral proteins and transgene products, noninflammatory causes, e.g., inactivation of promoter elements a



nflammatory
ene transfer
d-treated rats
t time points
nd (B) TNFα
TaqMan-RT
ues are given
housekeeping
significantly

tors, and up
increasing
supporting
ne response
ajor cause of
noviral gene
ne response
s, noninflam-
elements or

modifications of viral DNA, may also play a role. This was demonstrated in ex vivo cultures of adenovirally transfected corneas (*26*). We and others (*10, 11*) have shown that transgene expression was also limited in immunosuppressed animals, which suggests that noninflammatory causes might also play a role.

Similar kinetics of intragraft reporter gene expression were observed by others (*18–20*). In contrast to the delayed gene expression in heart graft models, a relatively fast expression was described in liver grafts (*21, 22*) which may reflect other experimental conditions or special properties of the liver tissue.

It has been shown that immunomodulatory approaches including anti-CD4 mAb treatment (*10–12*) may attenuate the specific immune response to ad-encoded proteins and prolong the transgene expression. Its efficiency for improving gene therapy in a transplant setting was not studied so far. We could demonstrate for the first time that a low-dose nondepleting anti-CD4 mAb approach combats the intragraft inflammatory response due to the synergistic action of ischemia/reperfusion and viral infection and mediate an earlier and longer reporter gene expression in a transplant setting. The faster and prolonged gene expression may improve the usefulness of gene therapy in transplantation as the modification of the early posttransplant phase strongly influence the long-term outcome of allografts.

The amplification of the *early* transgene expression by anti-CD4 mAb treatment was very surprising. The mechanisms of the early inflammation in ad-transfected grafts are not fully understood. Unspecific inflammatory events are discussed and the high intragraft IFNγ level at day 3 may be due to NK cell activity. However, the dramatic effects of CD4 targeting therapy on the early graft infiltration and the reduction of IFNγ expression by more than 80% redefines the role of $CD4^+$ cells (T cells and/or $CD4^+$ monocytes/macrophages) during the early antiviral response. Moreover, our data suggest that IFNγ may play an important role in down-regulating the CMV-promoter controlled expression of the transgene after in vivo gene transfer. Although the reporter gene expression was improved under the umbrella of anti-CD4 mAb as early as day 3, the further rise of $X\text{-gal}^+$ cells and total enzyme activity of the graft until day 7 suggests that other factors than IFNγ may be involved in early down-regulation. Further experiments should address this phenomenon.

Our data demonstrate that the nondepleting anti-CD4 mAb approach is also feasible for the *prolongation* of reporter gene expression under the special conditions of transplantation. The peak levels were higher and the ET_{50} of $X\text{-gal}^+$ cells increased from 18 to 32 days. For the interpretation of the two different methods used for measuring transgene expression it is important to note that the numbers of cells expressing β-gal were roughly similar in grafts of mAb-treated and mAb-untreated animals except days 28 and 56. However, in the ONPG-assay the differences between both groups have been significant until day 28. The early differences measured by the ONPG-assay might therefore rather be a result of altered transgene regulation than elimination of ad-infected cells which is also supported by the differences in IFNγ mRNA expression early after gene transfer. In contrast, at later time points (days 28 and 56) the elimination of ad-infected cells seems to play a more important role as seen the results obtained by the X-gal staining.

Even though $CD8^+$ CTL are major effectors in eliminati ad-infected cells, $CD4^+$ T cells also play an essential role the anti-ad immune response. It has been reported that ME class II-restricted activation of $CD4^+$ cells by ad-antigens necessary for complete activation of $CD8^+$ T cells (*23*). Mo over, $CD4^+$ cells alone seem to be capable of destroyi virus-infected hepatocytes (*23*). Additionally, $CD4^+$ cells a able to induce a humoral anti-ad immune response via ac vation of B cells. The important role of $CD4^+$ T cells for t elimination of transfected cells was further supported by t prolonged reporter gene expression in the lung followi application of depleting (*10*) or high-dose nondepleting (*1* anti-CD4 mAb. We could demonstrate that even a low-do nondepleting anti-CD4 mAb protocol can improve the expre sion of the gene of interest in the more complicated trar plant setting. The significant inhibition of cellular infiltr tion by anti-CD4 mAb may explain this effect. Howev although the cellular infiltration into the ad-transfect grafts kept at low levels during the whole observation tir (28 days) in anti-CD4 mAb-treated rats, the number of gal^- cells rapidly decreased after day 28. This may be due the development of a "low-level" ad-specific immune r sponse, which is reflected by a relatively high proportion $CD25^+$ cells among the infiltrating cells, particularly at da 3 and 7, compared with controls with nontransfected graf Additionally, the low-dose anti-CD4 therapy delayed a strongly reduced the anti-ad-antibody production. The tite measured in our experiments are consistent with results Chan et al. (*27*), who suggest that the titers in animals wi ad-perfused transplants were much lower than in mod using direct i. v. injection of adenoviral vectors. This may due to the extended circulation and presentation of adeno ral antigens in these models. If the reduced anti-ad antibo production by this low-dose anti-CD4 treatment might all a second vector administration should be further analyze

In summary, low dose nondepleting anti-CD4 mAb tre ment improved both the early and the late transgene expre sion in a model of syngeneic heart transplantation. The fore, this approach may be useful in future gene thera protocols in allotransplantation.

REFERENCES

1. Pellegrini C, O'Brien T, Yap J, Jeppsson A, Tazelaar HD, McG gor CGA. Systematic evaluation of distribution of transge expression after adenovirus-mediated gene transfer to t transplanted heart. Transplant Int 1998; 11: 373.
2. McClane SJ, Hamilton TE, Burke, CV, Raper SE. Functio consequences of adenovirus-mediated murine pancreatic ge transfer. Hum Gene Ther 1997; 8: 739.
3. Csete ME, Drazan KE, van Bree M, et al. Adenovirus-mediat gene transfer in the transplant setting. I. Conditions for e pression of transfered genes in cold-preserved hepatocyt Transplantation 1994; 57: 1502.
4. Kolls J, Peppel K, Silva M, Beutler B. Prolonged and effect blockade of tumor necrosis factor activity through adenovir mediated gene transfer. Proc Natl Acad Sci USA 1994; 91: 2
5. Yang Y, Li Q, Ertl HCJ, Wilson JM. Cellular and humo immune responses to viral antigens create barriers to lu directed gene therapy with recombinant adenoviruses. J Vi 1995; 69: 2004.
6. Yang Y, Nunes FA, Berencsi K, Furth EE, Gönczöl E, Wilson J

Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci USA 1994; 91: 4407.

7. Schmid C, Heemann U, Tilney NL. Factors contributing to the development of chronic rejection in heterotopic rat heart transplantation. Transplantation 1997; 64: 222.
8. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ Bromberg JS. Promoter attenuation in gene therapy: Interferon-γ and tumor necrosis factor-α inhibit transgene expression. Hum Gene Ther 1997; 8: 2019.
9. Ritter T, Schröder C, Risch K, et al. Ischemia/reperfusion injury-mediated downregulation of adenovirus-mediated gene expression in a rat heart transplantation model is inhibited by co-application of a TNFRp55-Ig chimeric construct. Gene Ther 2000; in press.
10. Kolls J K, Lei D, Odom GM, et al. Use of transient CD4 lymphocyte depletion to prolong transgene expression of E1-deleted adenoviral vectors. Hum Gene Ther 1996; 7: 489.
11. Ali RR, Reichel MB, Byrnes AP, et al. Co-injection of adenovirus expressing CTLA4-Ig prolongs adenovirally mediated LacZ reporter gene expression in the mouse retina. Gene Ther 1998; 5: 1561.
12. Lei D, Lehmann M, Shellito JE, et al. Nondepleting anti-CD4 antibody treatment prolongs lung-directed E1-deleted adenovirus-mediated gene expression in rats. Hum Gene Ther 1996; 7: 2273.
13. Lehmann M, Sternkopf F, Metz F, et al. Induction of long-term survival of rat skin allografts by a novel, highly efficient anti-CD4 monoclonal antibody. Transplantation 1992; 54: 959.
14. Volk HD, Siegling A, Platzer C, Brock J, Emmrich F, Lehmann M. Tolerance induction by anti-CD4 mab treatment in a rat kidney transplantation model. Transplant Proc 1994; 26: 3155.
15. Green MK. Rat heterotopic heart transplant technique. In: Green MK, Mandel TE, eds. Experimental transplantation models in small animals. Chur, Switzerland: Harwood Academic Publishers, 1995: 267.
16. MacGregor GR, Nolan GP, Fiering S, Roederer M, Herzenberg LA. Use of *E. coli* lacZ (β-galactosidase) as a reporter gene. In: Murray EJ ed. Methods in molecular biology. Vol. 7: gene transfer and expression protocols. Clifton, NJ: The Humana Press Inc., 1991: 217.
17. Held CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996; 5: 986.
18. Lee J, Laks H, Drinkwater DC, et al. Cardiac gene transfer by intracoronary infusion of adenovirus vector-mediated reporter gene in the transplanted mouse heart. J Thorac Cardiovasc Surg 1996; 111: 246.
19. Wang J, Ma Y, Knechtle SJ. Adenovirus-mediated gene transfer into rat cardiac allografts. Comparison of direct injection and perfusion. Transplantation 1996; 61: 1726.
20. Gojo S, Niwaya K, Taniguchi S, Nishizaki K, Kitamura S. Gene transfer into the donor heart during cold preservation for heart transplantation. Ann Thorac Surg 1998; 65: 647.
21. Shaked A, Csete ME, Drazan KE, et al. Adenovirus-mediated gene transfer in the transplant setting. II. Successful expression of transfered cDNA in syngeneic liver grafts. Transplantation 1994; 57: 1508.
22. Drazan K E, Wu L, Shen X, et al. Adenovirus-mediated gene transfer in the transplant setting. Part III. Variables affecting gene transfer in liver grafts. Transplantation 1995; 59: 670.
23. Yang Y, Wilson JM. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4$^+$ CTLs in vivo. J Immunol 1995; 155: 2564.
24. Tsui LV, Guidotti LG, Ishikawa T, Chisari FV. Posttranscriptional clearance of hepatitis B virus RNA by cytotoxic T lymphocyte-activated hepatocytes. Proc Natl Acad Sci USA 1995; 92: 12398.
25. Stein J, Volk HD, Liebenthal C, Krüger DV, Prösch S. TNF stimulates the activity of the human cytomegalovirus major immediate early enhancer/promoter in immature monocytic cells. J Gen Virol 1993; 74: 2333.
26. Oral HB, Larkin DFP, Fehervari Z, et al. Ex vivo adenovirus-mediated gene transfer and immunomodulatory protein production in human cornea. Gene Ther 1997; 4: 639.
27. Chan SY, Li K, Piecotti, JR, et al. Tissue-specific consequences of the anti-adenoviral immune response: implications for cardiac transplants. Nat Med 1999; 10: 1143.

Received 21 June 1999.
Accepted 9 February 2000.

EXHIBIT U

Tight Control of Exogenous SERCA Expression Is Required to Obtain Acceleration of Calcium Transients With Minimal Cytotoxic Effects in Cardiac Myocytes

J. Michael O'Donnell, Carlota M. Sumbilla, Hailun Ma, Iain K.G. Farrance, Marco Cavagna, Michael G. Klein, Giuseppe Inesi

Abstract—Collateral effects of exogenous sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) expression were characterized in neonatal rat and chicken embryo cardiac myocytes, and the conditions required to produce acceleration of Ca^{2+} transients with minimal toxicity were established. Cultured myocytes were infected with adenovirus vector carrying the cDNA of wild-type SERCA1, an inactive SERCA1 mutant, or enhanced green fluorescence protein under control of the cytomegalovirus promoter. Controls were exposed to empty virus vector. Each group was tested with and without phenylephrine (PHE) treatment. Under conditions of limited calf-serum exposure, the infected rat myocytes manifested a more rapid increase in size, protein content, and rate of protein synthesis relative to noninfected controls. These changes were not accompanied by reversal to fetal transcriptional pattern (as observed in hypertrophy triggered by PHE) and may be attributable to facilitated exchange with serum factors. SERCA virus titers >5 to 6 plaque-forming units per cell produced overcrowding of ATPase molecules on intracellular membranes, followed by apoptotic death of a significant number of rat but not chicken myocytes. Enhanced green fluorescence protein virus and empty virus also produced cytotoxic effects but at higher titers than SERCA. Expression of exogenous SERCA and enhancement of Ca^{2+} transient kinetics could be obtained with minimal cell damage in rat myocytes if the SERCA virus titer were maintained within 1 to 4 plaque-forming units per cell. Expression of endogenous SERCA was unchanged, but expression of exogenous SERCA was higher in myocytes rendered hypertrophic by treatment with PHE than in nontreated controls. **(*Circ Res.* 2001;88:415-421.)**

Key Words: SERCA ■ gene therapy ■ heart ■ adenovirus ■ calcium transients

The sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps Ca^{2+} from the cytosol back into the sarcoplasmic reticulum (SR) after myocardial contraction, thereby coordinating contractile tension and relaxation kinetics. Ca^{2+} uptake by the SR has been reported to be inadequate in failing human heart[1,2] as a consequence of reduced SERCA activity,[3] SERCA protein expression,[4,5] and SERCA mRNA levels.[6] On the other hand, it was shown in experimental models that uptake of cytosolic Ca^{2+} by the SR can be accelerated by expression of exogenous SERCA genes and consequent increase of the ATPase copy number in cardiac myocytes.[7–9] In fact, isolated failing human cardiac myocytes have shown improved performance after overexpression of exogenous SERCA.[10]

Recombinant adenovirus has proven to be a very effective vector for delivery of exogenous SERCA cDNA into cardiomyocytes,[11] with 100% efficiency of infection compared with 5% to 10% efficiency by other transfection methods.[12] The positive benefits of exogenous SERCA expression on Ca^{2+} homeostasis continues to be characterized by several laboratories, whereas collateral effects of gene expression have received little attention. We have observed important side effects that are much more evident in neonatal rat than in chicken embryo cardiac myocytes. In this study, we made comparative observations on cells infected with empty virus, with viral vectors carrying wild-type or inactive SERCA, or with enhanced green fluorescence protein (EGFP) cDNA. We describe here the effects of these procedures on protein synthesis, cell viability, and calcium handling in controls and hypertrophic (treated with phenylephrine [PHE]) myocytes. We then define restricted conditions under which the level of exogenous SERCA gene expression and improvement of cytosolic Ca^{2+} control can be obtained in rat myocytes with minimal cell damage.

Materials and Methods

DNA Constructs and Vectors

EGFP or wild-type or mutant chicken SERCA1[13] cDNA was subcloned into pAdlox[14] or pΔE1sp1A[15] plasmid. The cDNA was preceded by the cytomegalovirus (CMV) promoter and followed by simian virus polyadenylation signal. Recombinant adenovirus with EGFP or SERCA1 cDNA was obtained as previously described.[7,16]

Original received August 24, 2000; resubmission received December 12, 2000; accepted January 5, 2001.
From the Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Md.
Correspondence to Giuseppe Inesi, University of Maryland, 108 N Greene St, Baltimore, MD 21201-1503. E-mail ginesi@umaryland.edu

Circulation Research is available at http://www.circresaha.org

The recombinant products were selected by plaque purification in HEK293 cells and band-purified by centrifugation in cesium gradients to yield concentrations of the order of 10^9 to10^{11} plaque-forming units (pfu) per milliliter.

Preparation and Treatment of Neonatal Rat Myocytes

Chicken embryo cardiac myocytes were prepared and cultured as previously described.[7] Neonatal rat cardiac myocytes were prepared and cultured as follows.

Day 1

Primary cultures were obtained from 1-day-old Sprague-Dawley rats, as previously described,[16,17] and cultured in MEM (GibcoBRL) containing Hanks' salts, 5% calf serum, vitamin B_{12}, and 0.1 mol/L bromodeoxyuridine at 37°C, 350 cells/mm^2, in the presence of 1% CO_2. The culture medium was replaced every 12 hours throughout the experiment.

Day 2

The cells were washed, and the medium was replaced with half the original volume of serum-free medium (identical to that previously described except for the absence of serum). The cells were infected with adenovirus vector (0 to 20 pfu/cell) containing wild-type SERCA1, EGFP, or mutant cDNA encoding inactive SERCA1. Controls were exposed to empty virus. Infections were obtained by exposing the cells to the viral vectors for 1 hour. At this time, the medium was diluted 1:1 with medium containing serum to yield a final 5% concentration.

Day 3

Cell-culture medium was removed and replaced with a defined MEM containing 10 μg/mL transferrin, 10 μg/mL insulin, 0.1% BSA, 0.1 mol/L bromo deoxyuridine, 100 μmol/L vitamin C, and no serum. Two sets of plates (infected or noninfected) from day 2 were treated with 20 μmol/L PHE to induce hypertrophy.[17] Two sets of alternative plates were not treated with PHE.

Day 5

Multiple-phase contrast images of cell populations were taken from each plate to establish cell counts and viability. Both attached cells and dead cells (ie, floaters) were counted.

Cells were harvested for Western and Northern blots, [^{14}C]phenylalanine experiments, or fluorescence measurements of cytosolic calcium.

Cell Death and Protein Synthesis

The number of dead cells was estimated by counting attached cells and floaters in culture dishes. Total protein was measured by bicinchoninic acid assay (Pierce) after counting the cells in culture. Protein synthesis rate was measured using radioactive amino acid as described by Simpson.[17] To this aim, the culture media was brought to 0.1 μCi/mL [^{14}C]phenylalanine on day 3, and on day 5 [^{14}C]phenylalanine incorporation into the total cell protein was determined by scintillation counting.

Western and Northern Blot Analysis

SERCA protein content was determined by Western blots, as previously described.[12,16] Wild SERCA1 and inactive SERCA1 mutant were detected using primary antibodies CaF3-5C3[13] and Myc1-9E10 for the c-myc tag.[18] Endogenous SERCA2a was detected using MA3-919 antibody (Affinity Bioreagents). Atrial natriuretic factor (ANF), skeletal α-actin, and 18S mRNA levels were determined by Northern blots as described by Sumbilla et al.[12] cDNA probes for rat ANF and skeletal α-actin were radiolabeled with α[^{32}P]-dCTP using a random priming labeling kit (Amersham). The synthetic oligonucleotide probe for 18S mRNA was radiolabeled by terminal deoxynucleotide transferase with α[^{32}P]-dCTP.[19] Values for ANF and actin mRNA were normalized to endogenous 18S mRNA. In situ immunofluorescence staining of SERCA1 was performed as previously described.[7]

Intracellular Calcium Measurements

As described previously,[16] cell cultures were loaded with the Ca^{2+} indicator dye Fluo-4, mounted on an Olympus 1X70 inverted microscope, and superfused with Ringer's buffer solution at 30±2°C. The cells were field-stimulated, and cell fluorescence was recorded, corrected for background signal, and plotted as ΔF/Fo. Some experiments were conducted with cells loaded with the indicator dye Fura-2.[16]

Apoptosis

Apoptosis was assessed by microscopic visualization of condensed nuclei and electrophoretic demonstration of fragmented DNA patterns. For nuclear visualization, the cells were fixed with 4% paraformaldehyde (Sigma) and stained with 10 μg/mL Hoechst 33258 (Sigma B2283) in the presence of 0.1% Triton X-100 overnight in the dark at 4°C as previously described.[20] The stained cells were then visualized using an ultraviolet light (365 nm) on a Zeiss inverted microscope.

For demonstration of fragmented DNA pattern, DNA was isolated by phenol extraction and ethanol precipitation[21] and run on a 1% agarose gel. DNA patterns were compared with the classic fragmentation of DNA isolated from myocytes treated with staurosporin to induce apoptosis.[22]

Statistical Analysis

Experiments were done in triplicate. Data set comparisons were performed with Student's unpaired, 2-tailed *t*-test. Difference in mean values were considered statistically significant at $P<0.05$.

Results

Efficiency of Exogenous Gene Transfer in Neonatal Rat Cardiac Myocytes

In infections with adenovirus vectors, an important variable is the number of viral particles per cultured cell. We characterized this variable in our experiments by exposing neonatal rat cardiac myocytes to increasing titers (0 to 20 pfu/cell) of recombinant adenovirus vectors. It is important to realize that we indicate here viral titer with reference to pfu per attached, rather than seeded, cell to circumvent the variability of seeding rate in various experiments. Fluorescence images of cell cultures infected with EGFP virus revealed that 100% infection of rat myocytes is obtained with a viral titer of 5 pfu/cell (Figure 1A). Western blot analysis of SERCA1 expression revealed similar titer requirements. This pattern is different from that observed in chicken myocytes,[12] in which the titer required for 100% infection is 10 pfu/cell (Figure 1B).

Viral Infection and Cell Viability

Exogenous SERCA expression in neonatal rat cardiac myocytes decreases cell viability, resulting in detachment of a significant number of cells (floaters) even at viral titers as low as 5 pfu/cell (Figure 1A). This effect is observed to a much lesser extent in chicken-embryo cardiac myocytes (Figure 1B). It should be pointed out that an identical cytotoxic effect is produced by expression of wild-type SERCA1 or inactive SERCA mutant. Similar toxicity was observed after expression of SERCA2 (cDNA from either chicken or rabbit) in rat myocytes (not shown). EGFP virus (Figure 1A) and empty virus (not shown) also produce toxic effects. However, these effects are observed at significantly higher titer. It is clear that the range of viral titer between induction of protein expres-

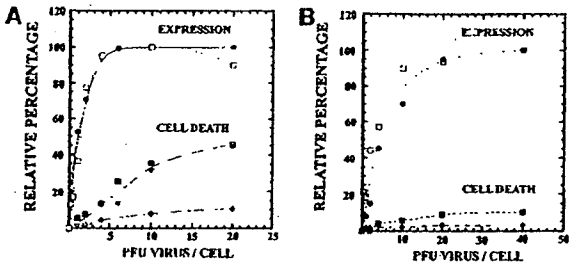


Figure 1. Exogenous gene expression or cell death after infection with increasing titer of adenovirus vectors. A, Neonatal rat cardiac myocytes. B, Chicken embryo cardiac myocytes. ●, percentage of cells exhibiting EGFP expression; □, levels of wild-type SERCA1 expression; ■, percentage of cell death after expression of SERCA1; ▽, inactive SERCA1 mutant; ◆, EGFP. EGFP was visualized by fluorescence microscopy, and SERCA protein was visualized by reaction with specific antibodies. Number of dead cells was determined by counting detached cells (floaters).

sion and production of toxicity is narrower for SERCA than for EGFP (Figure 1A).

Although we only use viral vectors derived from the first large-scale amplification of purified plaques, we considered whether the observed cytotoxic effects may be attributable to the presence of E1A and consequent replication of adenovirus in the infected cells. To rule out this possibility, we conducted parallel infections (1 pfu/cell) of A549 cells (able to amplify only replication-competent and not replication-defective virus) and HEK293 cells (E1A-transformed, used to amplify replication-defective virus) with SERCA1 adenovirus. We found no significant cell death in the A549 cells 3 days after infection, whereas 100% of the HEK293 cells were detached and obviously dead (Figure 2). Furthermore, plaque assays showed a 3 order of magnitude increase of plaque density in HEK293 cells and no increase in A549 cells and rat cardiac myocytes. This demonstrates that our stock of adenovirus vector does not contain E1A or replication-competent contaminants.

In situ immunofluorescence staining with antibodies specific for the exogenous SERCA1 reveals very dense packing of ATPase molecules within intracellular membranes even in seemingly healthy cells (Figure 3). Drastic structural changes are apparent in cells undergoing cytotoxic effects (Figure 3). It is noteworthy that cytotoxic effects are produced by wild-type SERCA and inactive SERCA mutant as well (Figure 1). It is likely that SERCA targeting of intracellular membranes and dense packing of ATPase molecules within a rather limited membrane space (Figure 3) produce perturbation of membrane structure and function and consequent alteration of calcium homeostasis. Furthermore, the occurrence of nuclear condensation and DNA fragmentation (Figure 4) suggests that apoptotic mechanisms, rather then necrosis, are involved in the cytotoxic effects of SERCA expression. The apoptotic index (percentage of nuclei exhibiting condensation) was 7% in myocytes infected with 2 pfu/cell and 31% in myocytes infected with 10 pfu/cell. It is noteworthy that similar apoptotic effects (Figure 4B) are also produced by higher titers of EGFP or empty virus.

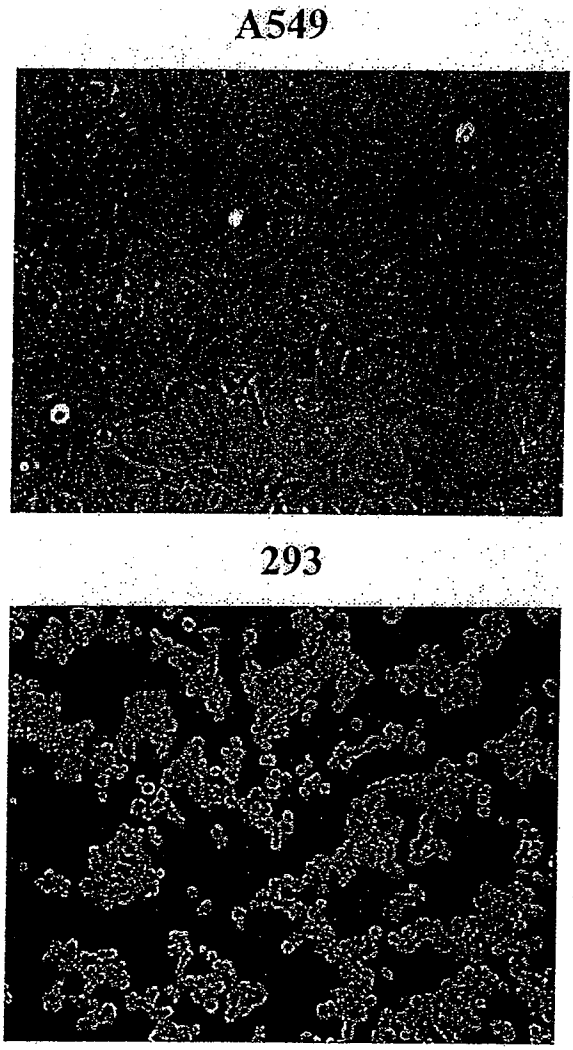


Figure 2. A549 and HEK293 cells infected with adenovirus vector carrying SERCA1 cDNA under control of the CMV promoter. Both cultures were infected with 1 pfu/cell, and the images shown above were obtained by phase-contrast microscopy 3 days after infection. Note that the (E1A-transformed) HEK293 cells undergo extensive cytotoxicity because of viral replication. On the other hand, the A549 cells remain perfectly healthy, demonstrating that the adenovirus vector used in these experiments lacks E1A and is replication-defective.

Cell Growth and Synthesis of Total and Specific SERCA Protein

An unexpected finding was that, under certain conditions, infection with adenovirus vector promotes increase in cell size and total protein synthesis. The magnitude of this effect is comparable to that of PHE. Figure 5 presents images of neonatal rat cardiac myocytes maintained with a defined medium in the absence of serum (Figures 5A and 5D), exposed to serum for 1 day (Figures 5B and 5E), or exposed continuously to serum (Figures 5C and 5F). Cells in Figures 5C through 5E were infected with SERCA1 virus. The cells were observed by phase-contrast microscopy 4 days after seeding. It is clear that the cells exposed continuously (Figures 5C and 5F) to serum are larger than the cells not exposed to serum (Figures 5A and 5D), independent of whether they were infected or not. This increase in cell size is

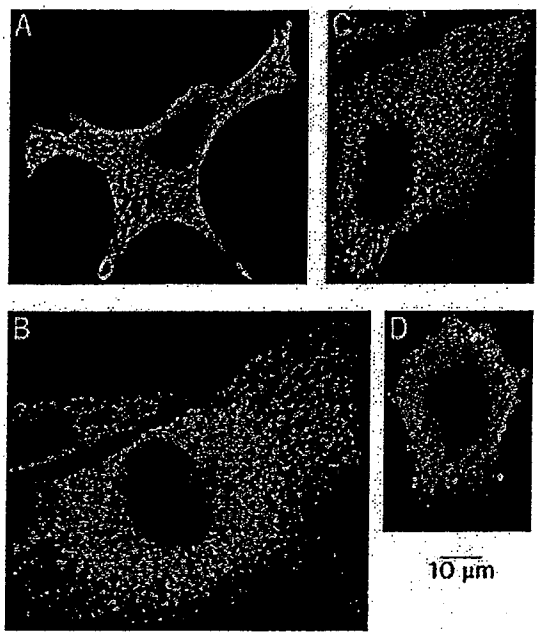


Figure 3. Intracellular membrane targeting of exogenous SERCA1 expression in neonatal rat cardiac myocytes and various cytotoxic stages. All cells were infected (4 pfu/attached cell) with adenovirus vector carrying SERCA1 cDNA under control of the CMV promoter. Exogenous SERCA was detected with specific monoclonal and fluorescent secondary antibodies. Selected myocytes show normal intracellular membrane network and dense packing of SERCA molecules within an apparently limiting membrane space (A, B, and C). A myocyte undergoing cytotoxic damage shows coalescence of intracellular membranes and rounded shape (D).

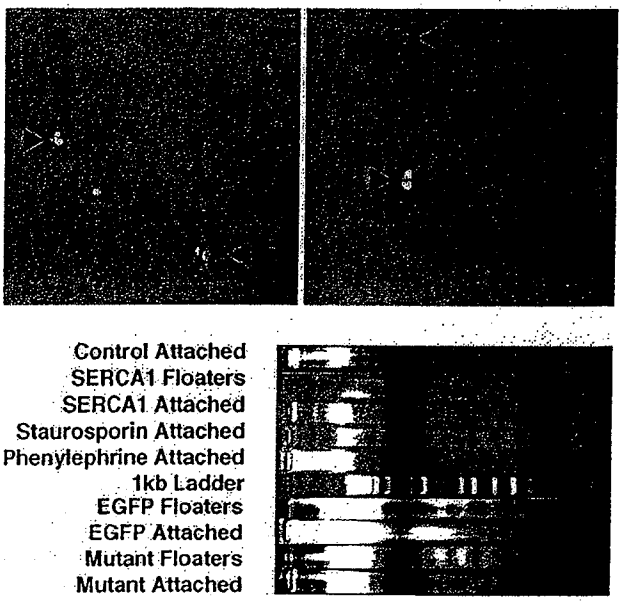


Figure 4. Nuclear condensation and DNA fragmentation in myocytes undergoing apoptosis as a consequence of exogenous SERCA overexpression. Top, Visualization of H&E-stained nuclei in infected myocytes. Bottom, Electrophoretic pattern of DNA extracted from control myocytes, attached myocytes, floaters infected with EGFP, wild-type SERCA1, or mutant SERCA1, and myocytes treated with staurosporine.[22] Note that even though DNA fragmentation is present in the EGFP floaters, the number of EGFP floaters is very low (Figure 1). Neonatal rat myocytes were infected (6 pfu/attached cell) with adenovirus vector.

comparable to that observed in cells treated with PHE (not shown). On the other hand, under conditions of limited exposure to serum, the size of infected cells is larger than that of noninfected cells (compare Figures 5B and 5E).

We next investigated whether the transcriptional pattern of cell growth and protein synthesis triggered by exogenous gene transfer was the same as that triggered by PHE. To this aim, we tested specific markers of α-adrenoceptor–mediated hypertrophy,[23–25] such as ANF and skeletal α-actin mRNA. We found these markers increased in myocytes treated with PHE but not at all increased in infected myocytes undergoing increase in size in the absence of PHE (Figure 6).

In all cases, the observed changes in cell size were matched by increased total protein content per cell (not shown) and rates of protein synthesis as revealed by radioactive phenylalanine incorporation (Figure 7A). In agreement with earlier studies,[17] we found that treatment with PHE for 48 hours resulted in a 70% increase in [^{14}C]phenylalanine incorporation, independent of viral infection. A similar increase was observed in the infected cells undergoing increase in size (compare control and infected cells in the absence of PHE, Figure 7A).

With regard to specific synthesis of SERCA, we found that endogenous SERCA2a is produced at the same level (per total protein unit weight) in control myocytes and myocytes undergoing PHE hypertrophy (Figure 7B). The endogenous SERCA2a level, however, is significantly reduced after infection with SERCA1 adenovirus (Figure 7B). This is likely attributable to competition with exogenous SERCA expression and membrane occupancy. In fact, we have previously shown[16] that even in noninfected myocytes, endogenous SERCA2a is quite densely spaced in the sarcoplasmic reticulum membrane. The total Ca^{2+}-dependent (thapsigargin-sensitive) ATPase (per total protein unit weight) is increased more than 3-fold in infected myocytes as a consequence of

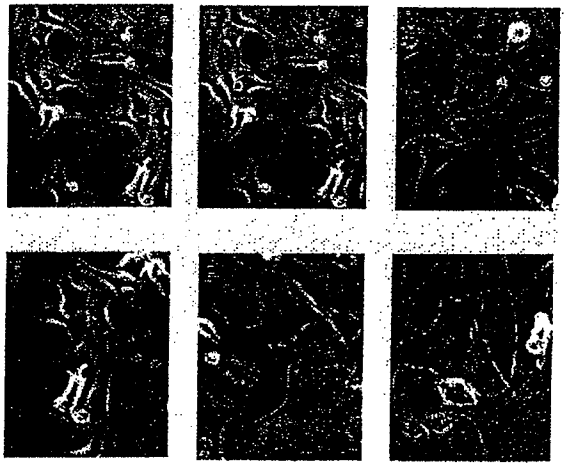


Figure 5. Control and infected neonatal rat cardiac myocytes maintained in the presence and absence of serum. A and D, Defined (no serum) medium for 4 days. B and E, Normal (with serum) medium for 1 day and defined (no serum) medium for 3 days. C and F, Normal (with serum) medium for 4 days. A, B, and C, control (noninfected) cells. D through F, Infection (2 pfu/cell) with SERCA1 adenovirus 1 day after seeding. All images were obtained by phase-contrast microscopy 4 days after seeding.

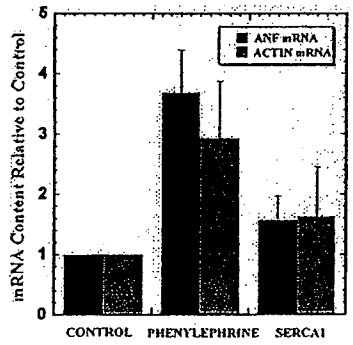


Figure 6. Analysis of transcriptional markers of hypertrophy in infected or PHE-treated myocytes. ANF Northern blot analysis of RNA samples isolated from cell cultures was used to identify the markers of hypertrophy. The blots were hybridized to [^{32}P]-labeled probes complementary to ANF and actin. When normalized to endogenous 18S mRNA, both ANF and skeletal α-actin increased in myocytes treated with PHE as expected. Infecting cells with either SERCA1 or EGFP virus resulted in only a modest increase of the 2 hypertrophy markers.

exogenous SERCA1 expression (Figure 7C). In fact, the rate of exogenous gene expression was higher in cells treated with PHE, as revealed by EGFP fluorescence and SERCA Western blots (results not shown). Consistent with these findings, the cytotoxic effect of SERCA expression was observed at lower titers in PHE-treated myocytes than in nontreated myocytes.

Exogenous SERCA Expression and Ca^{2+} Transients

The experiments reported above emphasize the importance of establishing conditions that limit exogenous SERCA expression to levels producing minimal cell damage while still improving the kinetics of Ca^{2+} transients. In a preliminary set of experiments, neonatal rat cardiac myocytes were infected with viral titers producing minimal cell damage (ie, 4 and 2 pfu/cell in the absence and presence of PHE, respectively). Ca^{2+} transients were then measured using the fluorescent Ca^{2+} indicator dye fluo-4 after a voltage stimulus. The data shown in Figure 8 were averaged from transients obtained from several cells selected at random (n=15 to 30). They demonstrate that a faster decay to baseline can be obtained after limited overexpression of exogenous SERCA in cells incubated either in the absence (Figure 8A) or in the presence (Figure 8B) of PHE. It is of interest that development of PHE-induced hypertrophy by itself does not significantly affect the Ca^{2+} transients (compare control and PHE transients in Figures 8A and 8B, respectively).

A series of measurements were also made with Fluo-4 in myocytes exposed to various viral levels, ranging between 0 and 10 pfu/seeded cell. Ca^{2+} transients were then obtained from several cells selected randomly in each plate. The time constants of decay and one-half width of the transients were averaged with the understanding that at viral titer <1 pfu/cell, the average values derive in part from infected and in part from noninfected cells. Nevertheless, it is clear from Figure 8C that the average decay constant of the entire cell population is significantly shortened after infection with 2 pfu/cell. This later titer produces minimal toxicity. Most importantly, Figure 8 shows that no additional reduction of the time constants is obtained by raising the viral titer above 2 pfu/cell.

We also obtained measurements with the indicator fura-2 and noted a modest reduction of the resting Ca^{2+} concentration in the cytosol of infected cells (24±5 nmol/L versus 42±7 nmol/L). On the other hand, no significant change in the peak Ca^{2+} concentration on stimulation after adequate rest was observed.[16] However, these measurements were obtained from healthy cells. Dead cells are supercontracted and often floating and are not suited to cytosolic calcium measurements.

Discussion

This study identifies important collateral effects of adenovirus vectors and gene transfer in neonatal rat cardiac myocytes as they relate to specific SERCA expression and modification of cytosolic Ca^{2+} transients. Cytotoxic effects, resulting in cell death, are most important collateral effects and are more prominently observed in neonatal rat than in chicken embryo myocytes. The difference in titer requirements may be a function of viral receptor density (related to species or tissue) as well as cell proliferation and density. Whereas the neonatal rat myocytes

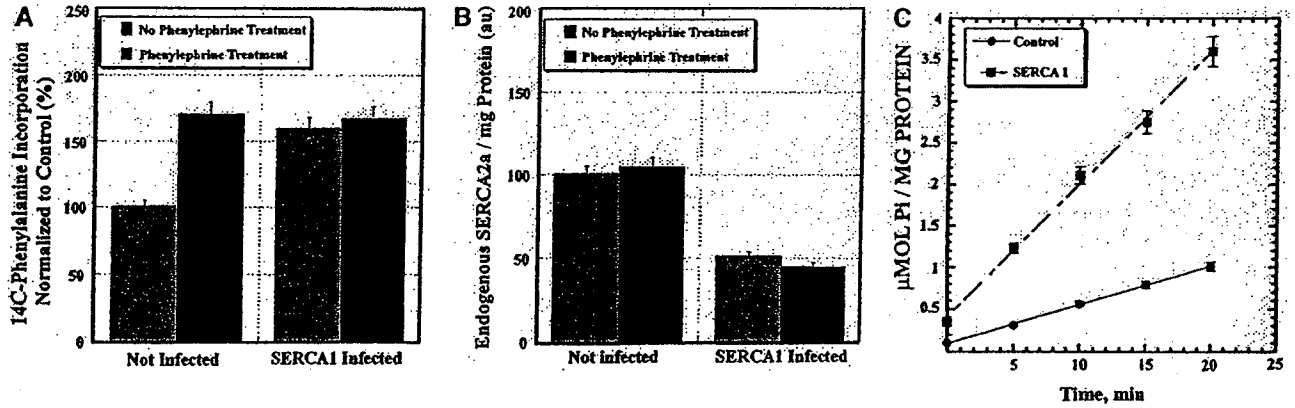


Figure 7. Total protein, endogenous SERCA2, and exogenous SERCA1 expression in neonatal cardiac myocytes in the absence and presence of PHE. A, [^{14}C]Phenylalanine incorporation as an index of total protein synthesis; the [^{14}C]phenylalanine pulse was added on day 3 after seeding. B, Endogenous SERCA2 expression as indicated by Western blots. C, Ca^{2+} ATPase (thapsigargin-sensitive) activity in control (noninfected) and infected cells. When indicated, cells were infected (4 pfu/cell) on day 2 after seeding, and PHE was added on day 2. In all cases, the cells were sampled on day 5.

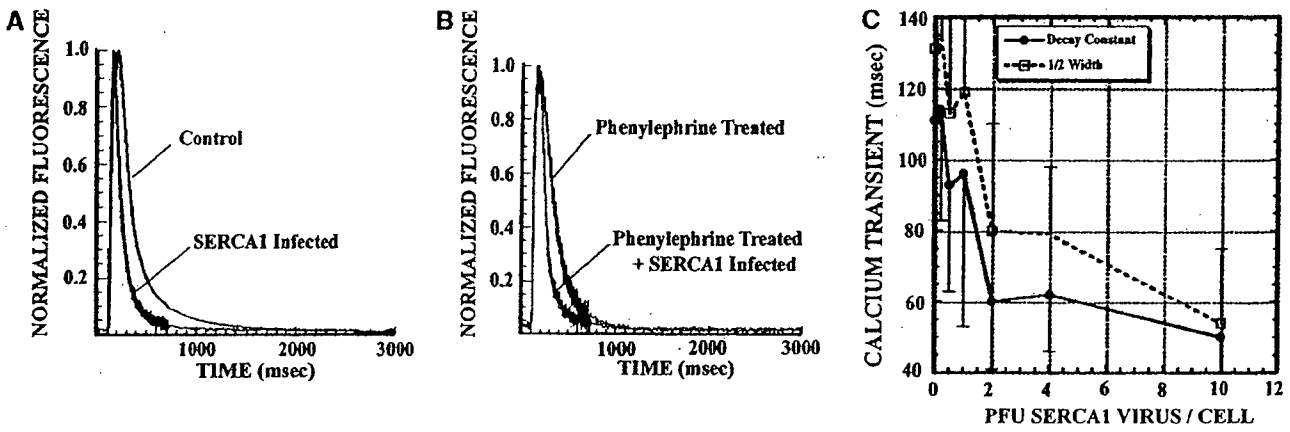


Figure 8. Effect of exogenous SERCA expression on cytosolic Ca^{2+} transients in neonatal rat cardiac myocytes. A and B, Averaged Ca^{2+} transients after repeated single-excitation pulses in noninfected and infected cells in the absence (A) and presence (B) of PHE-induced hypertrophy. When indicated, infection was performed with adenovirus carrying SERCA1 cDNA under control of the CMV promoter. The adenovirus titer was 4 pfu/cell in the absence of PHE and 2 pfu/cell in PHE-treated cells to account for the higher expression in these cells. C, Decay constants and half widths obtained in cells infected with increasing viral titer. Reported values were averaged from the Ca^{2+} transient of individual cells infected with SERCA1 virus. Cells in culture were selected at random for the measurements of Ca^{2+} transients. It is apparent that the Ca^{2+} transients of the entire cell population are optimally affected by a viral titer of 4 pfu/cell in the absence of PHE and 2 pfu/cell in the presence of PHE.

display limited cell division, the chicken embryonic myocytes proliferate significantly over the same period of time. We find that the entire population of rat myocytes is effectively infected with a viral titer of 2 to 5 pfu/attached cell, resulting in expression of SERCA levels that are sufficient to modify significantly the cytosolic Ca^{2+} transients. On the other hand, a significant percentage of rat myocytes receiving more than 2 to 4 pfu/cell undergo cytotoxicity and end up as floaters. The nuclear condensation and DNA fragmentation observed in our experiments are consistent with apoptotic death.[26] It is noteworthy that DNA fragmentation was also noted[27] in oncotic myocytes of infarct areas.

Because interference with cardiac gene transcription[28] and apoptotic effects[29,30] can be produced by adenovirus E1A, we made special efforts to exclude the presence of E1A and replication-competent virus in our preparation (Figure 2). Furthermore, the cytotoxic effects produced by EGFP or empty virus require a much higher titer than those produced by SERCA virus (Figure 1). It is likely that excessive expression and dense packing of membrane-bound SERCA molecules (Figure 3) damage the structural integrity of intracellular membranes. The consequent perturbation of membrane structure and function interferes then with intracellular calcium homeostasis. It is noteworthy that toxic effects are also observed with EGFP virus or empty virus if titers significantly higher are used.

Another collateral effect of viral infection is the increase in cell size and total protein synthesis observed under conditions of limited exposure to calf serum. This effect is in some cases of magnitude comparable to that of PHE hypertrophy but does not involve reversal to the fetal transcription pattern. The growth stimulus is evidently attributable to serum growth factors and is likely related to facilitated access from the medium to the cytosol in the infected myocytes. No effect of infection on growth is observed in the absence of serum, and maximal growth is observed independent of infection when the myocytes are continually exposed to serum. Awareness of this effect is likely to be helpful in studies of viral vectors and exogenous gene expression.

The optimal level of exogenous SERCA expression is clearly the viral titer that produces minimal toxic effects while achieving the desired functional response. This limit is 2 to 4 pfu/cell in neonatal rat cardiac myocytes expressing exogenous SERCA gene under control of the CMV promoter. This titer yields a 3-fold increase in SERCA activity and a pronounced kinetic effect on the cytosolic Ca^{2+} transients attributable to faster Ca^{2+} uptake by the sarcoplasmic reticulum. At higher viral titers, we observed no additional acceleration of Ca^{2+} transients but apoptotic death of a significant number of myocytes. It should be noted that we used the SERCA1 rather than the SERCA2 isoform, because SERCA1 has a higher turnover and can influence calcium transients with lower (and less toxic) levels of expression compared with SERCA2.[16]

An interesting alternative to our experiments of expression under control of the strong CMV promoter is the use of weaker promoters. We found that in this case, a higher viral titer is required to obtain SERCA expression levels that are effective on calcium transients. Consequently, no significant improvement in cytotoxicity is realized. Additional studies with promoters that may have the advantage of cell specificity as well as suitable strength are being conducted in our laboratory.

It is of interest that myocytes rendered hypertrophic by treatment with PHE increase their production of endogenous SERCA2a in proportion to total protein and retain unchanged Ca^{2+} transients. On the other hand, they react to adenovirus infection with faster expression of exogenous gene. Thereby, expression of exogenous SERCA and acceleration of Ca^{2+} transients, as well as cytotoxicity, are obtained at lower viral titers.

In conclusion, attempts to influence Ca^{2+} homeostasis by exogenous SERCA gene expression require careful control of protein expression levels and characterization of associated

cell functions. Failure to optimize conditions for adenovirus vector delivery and define collateral effects in various cell types is likely to create unwanted interference with progress in the experimental, and possibly therapeutic, use of this procedure.

Acknowledgments

This work was supported by the National Institutes of Health (NIH PO1-HL27867) and the University of Maryland Interdisciplinary Training Program in Muscle Biology (NIH 5T32-AR07592). J.M.O. received additional support from the Mid-Atlantic American Heart Association (Beginning Grant-in-Aid 0060286U).

References

1. Pieske B, Maier LS, Bers DM, Hasenfuss G. Ca^{2+} handling and sarcoplasmic reticulum Ca^{2+} content in isolated failing and nonfailing human myocardium. *Circ Res.* 1999;85:38–46.
2. Gwathmey JK, Copelas L, MacKinnon R, Schoen FJ, Feldman MD, Grossman W, Morgan JP. Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure. *Circ Res.* 1987; 61:70–76.
3. Schmidt U, Hajjar RJ, Helm PA, Kim CS, Doye AA, Gwathmey JK. Contribution of abnormal sarcoplasmic reticulum ATPase activity to systolic and diastolic dysfunction in human heart failure. *J Mol Cell Cardiol.* 1998;30:1929–1937.
4. Schotten U, Koenigs B, Rueppel M, Schoendube F, Boknik P, Schmitz W, Hanrath P. Reduced myocardial sarcoplasmic reticulum Ca^{2+}-ATPase protein expression in compensated primary and secondary human cardiac hypertrophy. *J Mol Cell Cardiol.* 1999;31:1483–1494.
5. Lehnart SE, Schillinger W, Pieske B, Pieske B, Prestle J, Just H, Hasenfuss G. Sarcoplasmic reticulum proteins in heart failure. *Ann N Y Acad Sci.* 1998;853:220–230.
6. Mercadier JJ, Lompre AM, Duc P, Boheler KR, Fraysse JB, Wisnewsky C, Allen PD, Komajda M, Schwartz K. Altered sarcoplasmic reticulum Ca^{2+}-ATPase gene expression in the human ventricle during end-stage heart failure. *J Clin Invest.* 1990;85:305–309.
7. Inesi G, Lewis D, Sumbilla C, Nandi A, Strock C, Huff KW, Rogers TB, Johns DC, Kessler PD, Ordahl CP. Cell-specific promoter in adenovirus vector for transgenic expression of SERCA 1 ATPase in cardiac myocytes. *Am J Physiol.* 1998;274: C645–C653.
8. Hajjar RJ, Kang JX, Gwathmey JK, Rosenzweig A. Physiological effects of adenoviral gene transfer of sarcoplasmic reticulum calcium ATPase in isolated rat myocytes. *Circulation.* 1997;95:423–429.
9. Giordano FJ, Huaping H, McDonough P, Meyer M, Sayen MR, Dillman WH. Adenovirus-mediated gene transfer reconstitutes depressed sarcoplasmic reticulum Ca^{2+} ATPase levels and shortens prolonged cardiac myocyte Ca^{2+} transients. *Circulation.* 1997;96:400–403.
10. del Monte F, Harding SE, Schmidt U, Matsui T, Kang ZB, Dec GW, Gwathmey JK, Rosenzweig A, Hajjar RJ. Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. *Circulation.* 1999;100:2308–2311.
11. Kirshenbaum LA. Adenovirus mediated-gene transfer into cardiomyocytes. *Mol Cell Biochem.* 1997;172:13–21.
12. Sumbilla C, Cavagna M, Zhong L, Ma H, Lewis D, Farrance I, Inesi G. Comparison of SERCA1 and SERCA2a expressed in COS-1 cells and cardiac myocytes. *Am J Physiol.* 1999;277:H2381–H2391.
13. Karin NJ, Kaprielian Z, Fambrough DM. Expression of avian Ca^{2+}-ATPase in cultured mouse myogenic cells. *Mol Cell Biol.* 1989;9: 1978–1986.
14. Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps M. Construction of adenovirus vectors through Cre-*lox* recombination. *J Virol.* 1997;71: 1842–1849.
15. Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. In: RW Ellis, ed. *Vaccines: New Approaches to Immunological Problems.* Woburn, Mass: Butterworth-Heinemann; 1992: 363–390.
16. Cavagna M, O'Donnell JM, Sumbilla C, Inesi G, Klein MG. Exogenous Ca^{2+} ATPase isoform effects on Ca^{2+} transients of embryonic chicken and neonatal rat cardiac myocytes. *J Physiol.* 2000;528:53–63.
17. Simpson P. Stimulation of hypertrophy of cultured neonatal rat heart cells through an α_1- and β_1-adrenergic receptor and induction of beating through an α_1- and β_1-adrenergic receptor interaction. *Circ Res.* 1985;56: 884–894.
18. Evans GI, Lewis GK, Ramsay G, Bishop JM. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Bio.* 1985;5:3610–3616.
19. O'Neill L, Holbrook NJ, Fargnoli J, Lakatta EG. Progressive changes from young adult age to senescence in mRNA for rat cardiac myosin heavy chain genes. *Cardioscience.* 1991;2:1–5.
20. Sheng Z, Knowlton K, Chen J, Hoshijima M, Brown JH, Chien KR. Cardiotrophin1 (CT-1) inhibition of cardiac myocytes apoptosis via a mitogen-activated protein kinase-dependent pathway: divergence from downstream CT-1 signals for myocardial cell hypertrophy. *J Biol Chem.* 1997;272:5783–5791.
21. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds. *Short Protocols in Molecular Biology.* 4th ed. New York, NY: John Wiley & Sons; 1999:2–3.
22. Yue TL, Wang C, Romanic AM, Kikly K, Keller P, DeWolf WE Jr, Hart TK, Thomas HC, Storer B, Gu JL, Wang X, Feurerstein GZ. Staurosporine-induced apoptosis in cardiomyocytes: a potential role of caspase-3. *J Mol Cell Cardiol.* 1998;30:495–507.
23. Bishopric NH, Simpson PC, Ordahl CP. Induction of the skeletal α-actin gene in α 1-adrenoceptor-mediated hypertrophy of rat cardiac myocytes. *J Clin Invest.* 1987;80:1194–1199.
24. Knowlton KU, Baracchini E, Ross RS, Harris AN, Henderson SA, Evans SM, Glembotski CC, Chien KR. Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during α-adrenergic stimulation of neonatal rat ventricular cells. *J Biol Chem.* 1991;266: 7759–7767.
25. Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med.* 1999;341:1276–1283.
26. Ma TS. Sarcoplasmic reticulum calcium ATPase overexpression induces cellular calcium overload and cell death. *Ann N Y Acad Sci.* 1998;853: 325–328.
27. Ohno M, Takemura G, Ohno A, Misao J, Hayakawa Y, Minatoguchi S, Fujiwara T, Fujiwara H. Apoptotic myocytes in infarct area in rabbit hearts may be myocytes with DNA fragmentation. *Circulation.* 1998;98: 1422–1430.
28. Kirshenbaum LA, Schneider MD. Adenovirus E1A represses cardiac gene transcription and reactivates DNA Synthesis in ventricular myocytes, via alternative pocket protein- and p300-binding domains. *J Biol Chem.* 1995;270:7791–7794.
29. Liu Y, Kitsis R. Induction of DNA synthesis and apoptosis in cardiac myocytes by E1A oncoprotein. *J Cell Biol.* 1996;133:325–334.
30. Bishopric NH, Zeng GQ, Sato B, Webster KA. Adenovirus E1A Inhibits cardiac myocyte-specific gene expression through its amino terminus. *J Biol Chem.* 1997;272:20584–20594.

EXHIBIT V

C11

Improvement in Survival and Cardiac Metabolism After Gene Transfer of Sarcoplasmic Reticulum Ca^{2+}-ATPase in a Rat Model of Heart Failure

Federica del Monte, MD, PhD; Eric Williams, BS; Djemal Lebeche, PhD; Ulrich Schmidt, MD, PhD; Anthony Rosenzweig, MD; Judith K. Gwathmey, VMD, PhD; E. Douglas Lewandowski, PhD; Roger J. Hajjar, MD

Background—In heart failure, sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) activity is decreased, resulting in abnormal calcium handling and contractile dysfunction. We have previously shown that increasing SERCA2a expression by gene transfer improves ventricular function in a rat model of heart failure created by ascending aortic constriction.

Methods and Results—In this study, we tested the effects of gene transfer of SERCA2a on survival, left ventricular (LV) volumes, and metabolism. By 26 to 27 weeks after aortic banding, all animals developed heart failure (as documented by >25% decrease in fractional shortening) and were randomized to receive either an adenovirus carrying the SERCA2a gene (Ad.SERCA2a) or control virus (Ad.βgal-GFP) by use of a catheter-based technique. Sham-operated rats, uninfected or infected with either Ad.βgal-GFP or Ad.SERCA2a, served as controls. Four weeks after gene transfer, survival in rats with heart failure treated with Ad.βgal-GFP was 9%, compared with 63% in rats receiving Ad.SERCA2a. LV volumes were significantly increased in heart failure (0.64±0.05 versus 0.35±0.03 mL, $P<0.02$). Overexpression of SERCA2a normalized LV volumes (0.46±0.07 mL) in the failing hearts. ^{31}P NMR analysis showed a reduced ratio of phosphocreatine to ATP content in failing+Ad.βgal-GFP compared with sham+Ad.βgal-GFP (0.82±0.13 versus 1.38±0.14, $P<0.01$). Overexpression of SERCA2a in failing hearts improved the phosphocreatine/ATP ratio (1.23±0.28).

Conclusions—In this study, we show that unlike inotropic agents that improve contractile function at the expense of increased mortality and worsening metabolism, gene transfer of SERCA2a improves survival and the energy potential in failing hearts. (*Circulation*. 2001;104:1424-1429.)

Key Words: gene therapy ■ heart failure ■ calcium ■ excitation ■ contractility

In cardiac muscle, both contraction and relaxation are intimately dependent on the function of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) pump, which is regulated by phospholamban. In congestive heart failure, deficiency in SERCA2a results in abnormal calcium handling and diminished contraction.[1,2] In addition, a decrease in phosphorylation of phospholamban has been reported in failing hearts, along with an increase in the phospholamban/SERCA2a ratio, contributing to the contractile dysfunction in heart failure.[3,4] These results are consistent with the model that a decrease in SERCA2a levels alters intracellular calcium homeostasis and contributes to contractile dysfunction in failing hearts.

Recently, we showed that restoration of SERCA2a to control levels in isolated failing human cardiomyocytes improved contraction and relaxation by correcting calcium handling.[5] Furthermore, in an animal model of heart failure, adenoviral gene transfer of SERCA2a improved contractile function in vivo, demonstrating the importance of SERCA2a as a therapeutic target.[6] Pharmacological agents that increase contractility, however, have been shown to worsen survival in patients with heart failure and to increase the energetic demand.[7] The heart requires a continuous supply of energy in the form of ATP by mostly oxidative metabolism, with the major energy reserve molecule represented by phosphocreatine (PCr).[8,9] In the normal heart, although the majority of the energy consumption is due to cross-bridge cycling, relaxation requires an energy expenditure of 15% to remove Ca^{2+} from the cytoplasm. This high level of energy required by SERCA2a reaction is directly related to the magnitude of

Received March 22, 2001; revision received June 1, 2001; accepted June 7, 2001.

From the Cardiovascular Research Center and the Cardiology Division, Massachusetts General Hospital (F.D., E.W., D.L., U.S., A.R., R.J.H.), and Harvard Medical School (J.K.G.), Boston, Mass; and the Department of Physiology and Biophysics, College of Medicine, University of Illinois at Chicago (E.D.L.).

Correspondence to Roger J. Hajjar, MD, Cardiovascular Research Center, Massachusetts General Hospital, 149 13th St. CNY-4, Charlestown, MA 02129. E-mail hajjar@cvrc.mgh.harvard.edu

Circulation is available at http://www.circulationaha.org

the Ca^{2+} gradient across the SR.[9] Failing hearts have a reduced PCr/ATP ratio, so less energy reserve is available for the cellular processes.

In this study, we tested the hypothesis that unlike currently used pharmacological agents that increase inotropy, reconstitution of normal levels of SERCA2a by adenoviral gene transfer would improve contractile performance as well as survival in aortic-banded rats that have developed heart failure without adversely affecting energetics.

Methods

Construction of Recombinant Adenoviruses

To construct the adenovirus containing SERCA2a cDNA, we used the method described by He et al,[10] whereby the backbone vector, containing most of the adenoviral genome (pAd.EASY1), is used and the recombination is performed in *Escherichia coli*. SERCA2a cDNA was subcloned into the adenoviral shuttle vector (pAd.TRACK), which uses the cytomegalovirus (CMV) long terminal repeat as a promoter. The shuttle vector used also has a concomitant green fluorescent protein (GFP) under the control of a separate CMV promoter. An adenovirus containing both β-galactosidase and GFP controlled by separate CMV promoters (Ad.βgal-GFP) was used as control. The adenoviruses were propagated in 293 cells. The titers of stocks used for these studies measured by plaque assays were 3×10^{11} pfu/mL for Ad.βgal-GFP and 1.8×10^{11} pfu/mL for Ad.SERCA2a, with particle/pfu ratios of 8:1 and 18:1, respectively. These recombinant adenoviruses were tested for the absence of wild-type virus by polymerase chain reaction of the early transcriptional unit E1.

Experimental Protocol

Four-week-old Sprague-Dawley rats (Charles River, Mass; 70 to 80 g) were anesthetized with pentobarbital (65 mg/kg IP) and placed on a ventilator. A suprasternal incision was made, exposing the aortic root, and a tantalum clip with an ID of 0.58 mm (Weck, Inc) was placed on the ascending aorta. Animals in the sham group underwent a similar procedure without insertion of a clip. The supraclavicular incision was then closed, and the rats were transferred back to their cages. The supraclavicular approach was performed because during gene delivery, a thoracotomy is necessary, and if the thorax is not opened during the initial aortic banding, adhesions are avoided when gene delivery is performed.

The animals were initially divided into 2 groups: 1 group of 45 animals with aortic banding and a second group of 42 animals that were sham-operated. Three animals in the aortic banding group did not survive the initial operation, and 2 animals in the sham-operated group did not survive. In the aortic-banded animals, we waited 26 to 28 weeks for the animals to develop left ventricular (LV) dilatation before cardiac gene transfer. In this last group as well as in the sham-operated group, 14 animals did not undergo gene transfer and were followed longitudinally. The rest of the animals underwent adenoviral gene transfer with either Ad.SERCA2a or Ad.βgal-GFP.

^{31}P NMR Measurements

Hearts were retrogradely perfused from a 100-cm hydrostatic perfusion column with modified Krebs-Henseleit buffer (mmol/L: NaCl 116, KCl 4, $CaCl_2$ 1.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, and $NaHCO_3$ 25, equilibrated with 95% O_2/5% CO_2 at 37°C) that contained 5 mmol/L glucose in a 2-L reservoir. Hearts beat spontaneously, contracting against a fluid-filled intraventricular balloon connected to a pressure transducer and inflated to an end-diastolic pressure of 5 mm Hg. A 10- to 15-mL volume of coronary effluent bathed the heart. A stable energetic state in rat hearts was confirmed from ^{31}P NMR signals of PCr, ATP, and inorganic phosphate (P_i) as previously described.[11] NMR data were collected on a Bruker 400-MHz spectrometer interfaced to a 9.4-T, vertical-bore, superconducting magnet. ^{31}P spectra were obtained from isolated hearts perfused within a broadband, 20-mm NMR probe (Bruker Instruments). ^{31}P NMR spectra were acquired in 128 scans with a 161-MHz, 45° excitation pulse, a 1.8-second repetition time, 35 ppm sweep width, and 8000 data points. Peak assignments were referenced to the well-established resonance signal of PCr at 0 ppm, with identification and assignment of the α-, β-, and γ-phosphate signals of ATP. Signal intensity was determined by NMR-dedicated data analysis.

Serial Echocardiographic Assessment

After 18 weeks of banding, serial echocardiograms were performed weekly in lightly anesthetized animals (pentobarbital 40 mg/kg IP). Transthoracic M-mode and 2D echocardiography was performed with a Hewlett-Packard Sonos 5500 imaging system with a 12-MHz broadband transducer. A mid–papillary level LV short-axis view was used, and measurements of posterior wall thickness, LV diastolic dimension, and fractional shortening were collected. Gene transfer was performed in all animals within 3 days of detection of a drop in fractional shortening of >25% compared with the fractional shortening at 18 weeks after banding. In the sham-operated rats, gene delivery was performed at 27 weeks.

Adenoviral Delivery Protocol

The group of animals subjected to aortic banding were further subdivided into 3 additional groups of 16, 12, and 14 receiving Ad.SERCA2a, Ad.βgal-GFP, or no adenovirus, respectively. The group of sham-operated animals was also subdivided into 3 groups of 14, 12, and 14 receiving Ad.SERCA2a, Ad.βgal-GFP, or no adenovirus. The adenoviral delivery system has been described previously by our group in detail.[11–13] Briefly, after the rats had been anesthetized and a thoracotomy performed, a 22-gauge catheter containing 200 mL of adenoviral solution (10^{10} pfu) was advanced from the apex of the LV to the aortic root. The aorta and main pulmonary artery were clamped for 20 seconds distal to the site of the catheter, and the solution was injected; then the chest was closed and the animals were allowed to recover.

Measurements of LV Volume and Elastance

Rats in the different treatment groups were anesthetized with 65 mg/kg of pentobarbital and mechanically ventilated. A 1.4F high-fidelity pressure transducer (Millar Instruments) was introduced into the LV. Four 0.7-mm piezoelectric crystals were placed over the surface of the LV along the short axis of the ventricle at the level of the mitral valve and at the apex of the LV to measure the intercrystal distances. The LV volume was derived by use of a mathematical model using Cardiosoft (Sonometrics Co). LV pressure-volume loops were generated under different loading conditions by clamping of the inferior vena cava. The end-systolic pressure-volume relationship was obtained by producing a series of pressure-volume loops and connecting the upper left corners of the individual pressure-volume loops to generate the maximal slope.

Western Blot Analysis and SERCA2a Activity

The preparation of lysates was described earlier. Briefly, lysates were prepared at 4°C in a lysis buffer containing (in mmol/L) NaCl 150, $MgCl_2$ 1, and $CaCl_2$ 1, plus detergents and proteinase inhibitors (pH 7.4). The tissue was homogenized and spun at 1400 rpm (Sorvall) for 30 minutes. The supernatant was then filtered through 4 layers of gauze and centrifuged at 15 000 rpm for 60 minutes (Beckman). SDS-PAGE was performed on the supernatant under reducing conditions on 7.5% separation gels with a 4% stacking gel in a Miniprotean II cell (Biorad). For immunoreaction, the blots were incubated with 1:2500 diluted monoclonal antibodies to SERCA2a (MA3-919; Affinity BioReagents), phospholamban (Upstate Biotechnology), or 1:1000 diluted anti-calsequestrin (MA3-913; Affinity Bioreagents) for 90 minutes at room temperature.

Crude membranes were prepared as described by Schwinger et al[9] at 4°C in a buffer containing (in mmol/L) sucrose 300, PMSF 1, and PIPES 20, pH 7.4. The tissue was homogenized and spun at 8000 rpm (Beckman JA 20) for 20 minutes. The supernatant was then filtered through 4 layers of gauze and centrifuged at 35 000 rpm for 60 minutes (Sorvall). The pellet was resuspended in a 10% sucrose

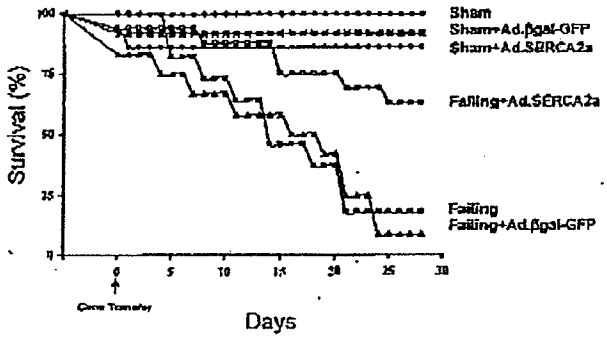


Figure 1. Survival-function curve of 6 different groups studied: sham, n=14; sham+Ad.βgal-GFP, n=12; sham+Ad.SERCA2a, n=14; failing, n=14; failing+Ad.βgal-GFP, n=12; failing+SERCA2a, n=16.

buffer containing (in mmol/L) KCl 400, $MgCl_2$ 0.5, $CaCl_2$ 0.5, EGTA 0.5, and PIPES 25, pH 7.0. SERCA2a activity assays were carried out on the basis of a pyruvate/NADH coupled reaction as previously described.[13,14] Ca^{2+}-ATPase activity was calculated as Δabsorbance/(6.22×protein×time) in nmol ATP/(mg protein×min).

Statistics

All values are presented as mean±SD. A 2-factor ANOVA was performed to compare the different hemodynamic parameters among the different groups. For the echocardiography data, when the variables were examined at various intervals, ANOVA with repeated measures was performed. Comparison of survival in the different groups of animals was analyzed by a log-rank test with the Kaplan-Meier method. Statistical significance was accepted at the level of $P<0.05$.

Results

Survival

Figure 1 shows the survival curve for the 6 different groups studied. The sham-operated animals did not show any premature mortality. The sham-operated animals that were infected with either Ad.βgal-GFP or Ad.SERCA2a had early mortalities related to the surgical intervention, but then the survival curves leveled off for both sham+Ad.βgal-GFP and sham+Ad.SERCA2a. In the failing group, the noninfected animals had a survival curve that decreased steadily, and at 4 weeks the survival rate was only 18% ($P<0.0005$ compared with sham). In the failing+Ad.βgal-GFP group, the survival curve also decreased, and at 4 weeks the survival rate was only 9% ($P<0.001$ compared with sham+Ad.βgal-GFP). In the failing+Ad.SERCA2a group, however, the survival curve was significantly improved compared with failing+Ad.SERCA2a ($P<0.001$ compared with failing+Ad.βgal-GFP).

Characterization of Animals

After 18 weeks of aortic banding, the animals showed echocardiographic signs of LV hypertrophy, including an increase in wall thickness (both posterior and septal), an increase in posterior wall thickness, a decrease in LV dimensions, and an increase in fractional shortening, as shown in Table 1. After 26 to 27 weeks of banding, these animals had uniformly (1) small pericardial effusions, (2) pleural effusions, (3) an increase in lung weight, (4) ascites, and (5) dyspnea at rest, all indicative signs of severe heart failure. Echocardiographically, LV end-diastolic dimensions increased and fractional shortening decreased.

Cardiac Gene Transfer and SERCA2a Expression

Protein levels of SERCA2a were decreased in failing compared with sham LVs, as shown in Figure 2A. Adenoviral gene transfer of SERCA2a in failing hearts increased SERCA2a protein expression, restoring it to levels observed in the nonfailing hearts. Calsequestrin did not change among the different groups, nor did phospholamban. As shown in Figure 2B, tabulated ratios of SERCA2a to phospholamban and SERCA2a to calsequestrin reveal a significant decrease in failing hearts and a restoration to control levels with gene transfer of SERCA2a.

SERCA2a Activity

We measured SERCA2a activity at a calcium concentration of 10 mmol/L in the (1) sham+Ad.βgal-GFP, (2) failing+Ad.βgal-GFP, and (3) failing+Ad.SERCA2a groups. There was a decrease in maximal ATPase activity in the failing group (27.4±4.9 versus 62.2±12.8 nmol · mg^{-1} · min^{-1}). Gene transfer of SERCA2a restored ATPase activity back to normal levels in the failing group 4 weeks after gene transfer (61.0±8.5 nmol · mg^{-1} · min^{-1}).

NMR Spectroscopy

Representative ^{31}P NMR spectra obtained from 3 groups of rats: (1) sham+Ad.βgal-GFP, (2) failing+Ad.βgal-GFP, and (3) failing+Ad.SERCA2a, are shown in Figure 3A. These spectra show that the ratios of total amounts of PCr to ATP are lower in the failing heart than the sham heart. The integrated area for P_i was also increased in the failing heart.

TABLE 1. Echocardiographic Measures in Rats After Sham Surgery or Aortic Banding

	Septum, mm	PW, mm	LVEDD, mm	LVESD, mm	FS, %
Sham	14.9±1.1	13.5±1.0	66.8±3.8	40.4±6.0	40.0±6.3
Aortic banding (18 weeks)	20.1±3.9‡	19.8±2.6‡	61.9±6.4*‡	34.0±6.2*‡	46.0±8.2*‡§
Aortic banding (27 weeks)	19.7±2.8†	18.5±2.3†	69.5±6.3§	45.1±6.9‡	36.0±10.4§

PW indicates posterior wall thickness during diastole; LVEDD, LV diameter at end diastole; LVESD, LV diameter at end systole; and FS, fractional shortening.
*$P<0.0005$ vs aortic banding (27 weeks).
†$P<0.005$, ‡$P<0.005$, §$P<0.05$ vs sham.

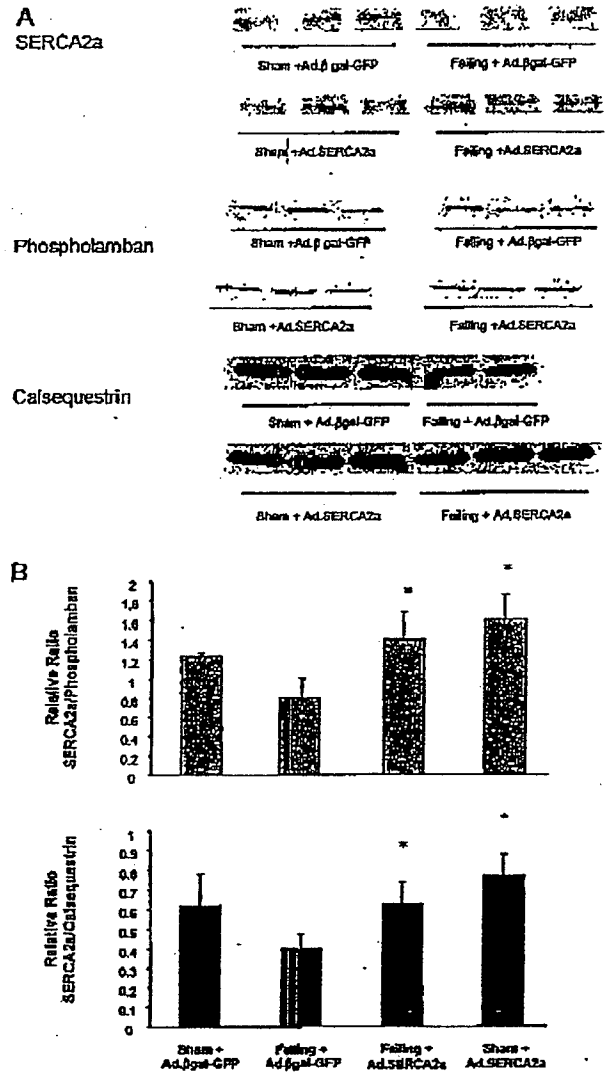


Figure 2. A, Immunoblots of SERCA2a, phospholamban, and calsequestrin from crude membranes of LVs from sham rats infected with Ad.βgal-GFP, failing rat hearts infected with Ad.βgal-GFP, and failing rats infected with Ad.SERCA2a. B, Relative protein levels of SERCA2a normalized to either phospholamban or calsequestrin ($n=6$ in all groups). *$P<0.05$ vs sham+Ad.βgal-GFP.

The overexpression of SERCA2a in failing heart restored and normalized the content of both PCr and ATP (Figure 3B). Interestingly, we found that overexpression of SERCA2a in sham-operated animals induces a reduction in PCr/ATP ratio.

Effects of SERCA2a Overexpression on Pressure-Volume Relationship

Pressure-volume analysis was performed in a subset of animals. LV volumes were significantly increased in the failing rats (0.64±0.05 versus 0.35±0.03 mL, $P<0.02$) and were decreased after SERCA2a gene transfer (0.46±0.07 mL). The slope of the end-systolic pressure-volume relationship (Figure 4) was lower in failing hearts infected with Ad.βgal-GFP ($n=5$) than in sham ($n=6$), indicating a diminished state of intrinsic myocardial contractility: 450±71 versus 718±83 mm Hg/mL ($P<0.02$). Gene transfer of SERCA2a restored the slope of the end-systolic pressure-volume relationship to control levels (691±91 mm Hg/mL,

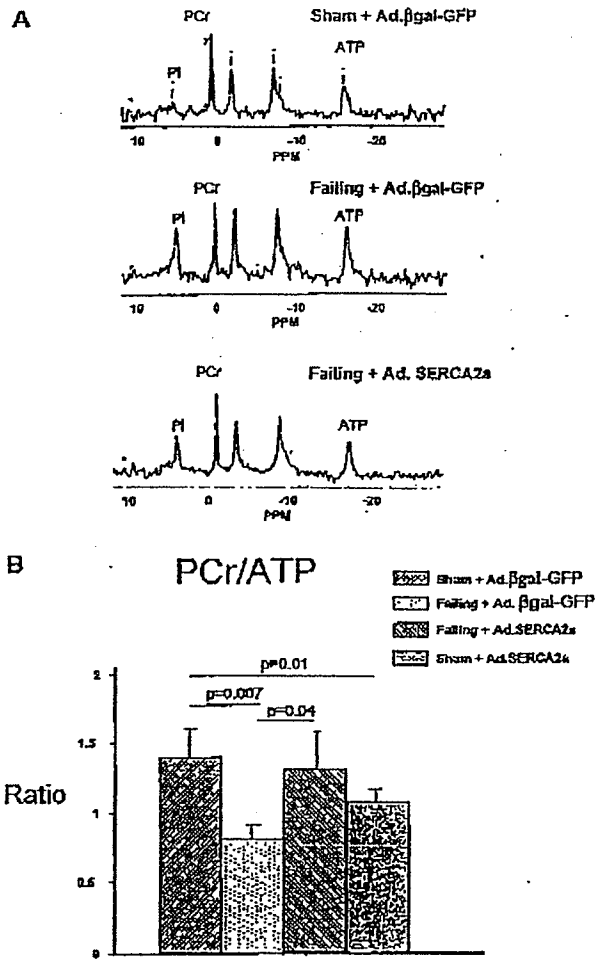


Figure 3. A, Representative ^{31}P-NMR spectra of sham+Ad.βgal-GFP, failing+Ad.βgal-GFP, and failing+Ad.SERCA2a hearts. Major resonances are assigned as P_i, PCr, and α-, γ-, and β-phosphates of ATP. Integrated peak intensities are proportional to metabolite content. B, Failing spectrum illustrates that PCr-to-ATP ratio and PCr and ATP contents in failing heart are lower than in sham heart. In spectrum of failing+Ad.SERCA2a heart, PCr-to-ATP ratio is restored toward normal.

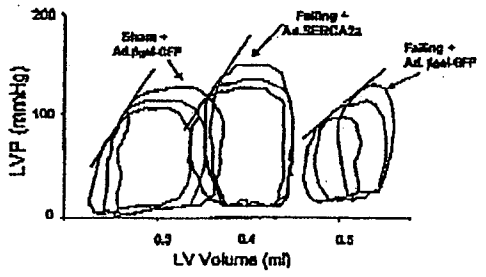


Figure 4. LV pressure (LVP) vs LV volume detected by piezoelectric crystals in a sham+Ad.βgal-GFP heart, a failing+Ad.βgal-GFP heart, and a failing+Ad.SERCA2a heart.

TABLE 2. Morphometric Analyses

	Sham+Ad.βgal-GFP	Sham+Ad.SERCA2a	Failing+Ad.βgal-GFP	Failing+Ad.SERCA2a
HW/BW×10⁴	3.7±0.3	4.4±0.6	4.4±0.5*	4.3±0.4*
HW/TL×10², g/mm	44.8±4.3	55.3±6.2	50.8±4.4*	50.3±6.3*

HW indicates heart weight; BW, body weight; and TL, tibial length.
*$P<0.05$ vs Sham+Ad.GFP.

n=6, $P<0.03$ versus failing+Ad.βgal-GFP; $P>0.1$ versus sham+Ad.βgal-GFP).

Effect on Morphological Parameters

As shown in Table 2, the failing hearts had a significant increase in heart weight when normalized to either tibial length or body mass. Gene transfer of SERCA2a in the failing heart did not have a significant effect on LV mass whether normalized to tibial length or body mass.

Discussion

In this study, we show that in an animal model of heart failure and contractile dysfunction, restoration of SERCA2a expression by cardiac gene transfer in vivo improves not only contractile function but also survival and cardiac energetics.

Abnormal SR Function and Cardiac Gene Transfer of SERCA2a

Impaired SERCA2a activity is one of the main characteristics associated with abnormal calcium handling in heart failure.[1,2] A decrease in SERCA2a relative to phospholamban and a reduction of phosphorylation of phospholamban contribute to the contractile dysfunction in human heart failure.[3,4] More recently, the ratio of the Na/Ca exchanger to SERCA2a has been shown to be increased in failing hearts and to be predictive of diastolic function in these hearts.[15] Gene transfer of SERCA2a corrects both the SERCA2a/phospholamban ratio and the SERCA2a/Na/Ca ratio and would contribute to restoring both systolic and diastolic function.

In our study, we showed that SERCA2a protein levels were restored to normal levels in the failing hearts and that this effect was sustained for up to 4 weeks. This seemed somewhat surprising, because first-generation adenoviruses induce transient expression peaking at 7 to 10 days and disappearing after 10 days.[16] Endogenous turnover of SERCA2a, however, is ≈14 to 15 days in young rats and longer in older rats,[17] which would explain the sustained levels of SERCA2a.

SERCA2a Expression and Cardiac Energetics

Decreased energy reserve via the creatine kinase reaction is a characteristic finding in both human and experimental heart failure.[9,18,19] This decrease in energy reserve contributes to the development of contractile dysfunction in heart failure.[19] In addition, an increase in intracellular P_i has been shown to reduce SR calcium loading and to depress calcium-induced calcium release.[20] Local ATP regeneration by the creatine kinase system is one mechanism the cell can use to improve Ca^{2+} uptake by the SR in conditions in which an excessive increase in cytoplasmic $[Ca^{2+}]$ may have deleterious effects. Recently, Tian et al[9] showed that pharmacological inhibition of creatine kinase resulted in altered energetics and induced abnormal Ca^{2+} handling and contractile dysfunction in the rat. In our experiments, restoring SERCA2a levels to normal induced an improvement in the ratio of PCr to ATP.

The findings of improved cardiac energetics in heart failure was somewhat surprising, because an increase in contractility by SERCA2a overexpression would be anticipated to increase ATP hydrolysis, thereby driving PCr down. Indeed, this increase in ATP hydrolysis is consistent with our observation of reduced PCr/ATP in the group of sham-operated hearts that were overexpressing SERCA2a. These results are also consistent with previous results showing that PCr/ATP was decreased in the phospholamban-deficient hearts relative to the wild-type hearts.[21]

In heart failure, however, elevated calcium levels would increase energy demand. To maintain low levels of diastolic Ca^{2+}, a high level of free energy released from ATP hydrolysis ($|\Delta Gp|$) is necessary. To maintain the normal Ca^{2+} gradient (≈10 000-fold between the cytosol and the SR), the SERCA2a reaction requires a $|\Delta Gp|$ of ≥52 kJ/mol, 85% to 90% of it from ATP.[9] Therefore, of all the ATPase reactions in cardiac myocytes, the SERCA2a reaction is the most vulnerable to a decrease in $|\Delta Gp|$.

Survival After Gene Transfer: Therapeutic Implications

In this model of heart failure, SERCA2a overexpression improved parameters of inotropy and normalized contractile reserve. These effects translate into an inotropic intervention. Other inotropic interventions, however, have been shown clinically to increase mortality.[22] There are, however, significant differences between enhancing inotropy with pharmacological agents that usually increase cAMP and gene transfer of SERCA2a. Unlike agents that increase cAMP, thereby increasing intracellular Ca^{2+}, restoration of SERCA2a levels decreases diastolic Ca^{2+}. Furthermore, it has been shown that sustained elevations of resting Ca^{2+} lead to activation of serine-threonine phosphatases, including calcineurin, inducing hypertrophy and cell death.[23] Therefore, a decrease in diastolic Ca^{2+} may in effect reduce the proapoptotic and prohypertrophy signaling. Heart failure is associated with an increased incidence of ventricular arrhythmias, and triggered activity is a probable mechanism of arrhythmogenesis in heart failure. The increase in intracellular calcium secondary to SERCA2a downregulation increases the arrhythmogenic potential. Preventing an increase in intracellular calcium by overexpression of SERCA2a prevents the induction of triggered activity. Furthermore, improvement in energetics is another important finding in this study that may have a direct influence on survival.

Conclusions

Our results demonstrate that restoring SERCA2a expression can improve not only systolic and diastolic performance in

failing hearts but also survival and cardiac energetics. Furthermore, SERCA2a normalization halts the adverse remodeling that occurs with congestive heart failure. This study validates the feasibility of cardiac gene transfer in failing hearts as a therapeutic modality.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health: HL-50361 and HL-57623 (Dr Hajjar); HL-59521 and HL-61557 (Dr Rosenzweig); HL-49574 (Dr Gwathmey); and HL-62702 (Dr Lewandowski); and a Doris Duke Charitable Foundation Clinician Scientist Award and American Federation of Aging research grant (Dr Hajjar). Dr Rosenzweig is an Established Investigator of the American Heart Association.

References

1. Schmidt U, Hajjar RJ, Helm PA, et al. Contribution of abnormal sarcoplasmic reticulum ATPase activity to systolic and diastolic dysfunction in human heart failure. *J Mol Cell Cardiol.* 1998;30:1929–1937.
2. Gwathmey JK, Copelas L, MacKinnon R, et al. Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure. *Circ Res.* 1987;61:70–76.
3. Schwinger RH, Bohm M, Schmidt U, et al. Unchanged protein levels of SERCA II and phospholamban but reduced Ca^{2+} uptake and Ca^{2+}-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. *Circulation.* 1995;92:3220–3228.
4. Schwinger RH, Munch G, Bolck B, et al. Reduced Ca^{2+}-sensitivity of SERCA 2a in failing human myocardium due to reduced serin-16 phospholamban phosphorylation. *J Mol Cell Cardiol.* 1999;31:479–491.
5. del Monte F, Harding SE, Schmidt U, et al. Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. *Circulation.* 1999;100:2308–2311.
6. Miyamoto MI, del Monte F, Schmidt U, et al. Adenoviral gene transfer of SERCA2a improves left-ventricular function in aortic-banded rats in transition to heart failure. *Proc Natl Acad Sci USA.* 2000;97:793–798.
7. O'Connor CM, Gattis WA, Uretsky BF, et al. Continuous intravenous dobutamine is associated with an increased risk of death in patients with advanced heart failure: insights from the Flolan International Randomized Survival Trial. *Am Heart J.* 1999;138:78–86.
8. Lewandowski ED, Damico LA, White LT, et al. Cardiac responses to induced lactate oxidation: NMR analysis of metabolic equilibria. *Am J Physiol.* 1995;269:H160–H168.
9. Tian R, Halow JM, Meyer M, et al. Thermodynamic limitation for Ca^{2+}-handling contributes to decreased contractile reserve in rat hearts. *Am J Physiol.* 1998;275:H2064–H2071.
10. He TC, Zhou S, da Costa LT, et al. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA.* 1998;95:2509–2514.
11. Hajjar RJ, Schmidt U, Matsui T, et al. Modulation of ventricular function through gene transfer in vivo. *Proc Natl Acad Sci USA.* 1998;95:5251–5256.
12. Hajjar RJ, del Monte F, Matsui T, et al. Prospects for gene therapy for heart failure. *Circ Res.* 2000;86:616–621.
13. Miyamoto MI, del Monte F, Schmidt U, et al. Adenoviral gene transfer of SERCA2a improves left-ventricular function in aortic-banded rats in transition to heart failure. *Proc Natl Acad Sci USA.* 2000;97:793–798.
14. Schmidt U, Hajjar RJ, Kim CS, et al. Human heart failure: cAMP stimulation of SR Ca^{2+}-ATPase activity and phosphorylation level of phospholamban. *Am J Physiol.* 1999;277:H474–H480.
15. Hasenfuss G, Schillinger W, Lehnart SE, et al. Relationship between Na^+-Ca^{2+}-exchanger protein levels and diastolic function of failing human myocardium. *Circulation.* 1999;99:641–648.
16. Gutman RJ, Lemarchand P, Crystal RG, et al. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. *Circ Res.* 1993;73:1202–1207.
17. Martonosi A, Halpin RA. Sarcoplasmic reticulum. 17: the turnover of proteins and phospholipids in sarcoplasmic reticulum membranes. *Arch Biochem Biophys.* 1972;152:440–450.
18. Neubauer S, Horn M, Cramer M, et al. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. *Circulation.* 1997;96:2190–2196.
19. Lino R, Nascimben L, Friedrich J, et al. Decreased energy reserve in an animal model of dilated cardiomyopathy: relationship to contractile performance. *Circ Res.* 1996;78:893–902.
20. Smith GL, Duncan AM, Neary P, et al. P_i inhibits the SR Ca^{2+} pump and stimulates pump-mediated Ca^{2+} leak in rabbit cardiac myocytes. *Am J Physiol.* 2000;279:H577–H585.
21. Chu G, Luo W, Slack JP, et al. Compensatory mechanisms associated with the hyperdynamic function of phospholamban-deficient mouse hearts. *Circ Res.* 1996;79:1064–1076.
22. Stevenson LW. Inotropic therapy for heart failure. *N Engl J Med.* 1998;339:1848–1850.
23. Lim HW, Molkentin JD. Calcineurin and human heart failure. *Nat Med.* 1999;5:246–247.

EXHIBIT W

Gene Therapy (2003) 10, 1807–1813

www.nature.com/gt

Efficient and long-term intracardiac gene transfer in δ-sarcoglycan-deficiency hamster by adeno-associated virus-2 vectors

J Li[1], D Wang[2], S Qian[3], Z Chen[3], T Zhu[1,2] and X Xiao[1,4]

[1]*Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, PA, USA;* [2]*Tongji Hospital, Huazhong Science and Technology University, Wuhan, China;* [3]*Department of Surgery, Thomas E Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, PA, USA; and* [4]*Department of Orthopedic Surgery, University of Pittsburgh, Pittsburgh, PA, USA*

Intracardiac gene transfer and gene therapy have been investigated with different vector systems. Here we used adeno-associated virus (AAV) vectors to deliver either a reporter gene or a therapeutic gene into the heart of golden Syrian hamsters. The method of gene delivery was direct infusion of the AAV2 vectors into the coronary artery ex vivo in a heterotopically transplanted heart. When an AAV2 vector carrying the Lac-Z gene driven by CMV promoter was delivered into the heart of healthy hamsters, effective gene transfer was achieved in up to 90% of the cardiomyocytes. Lac-Z gene expression persisted for more than 1 year without immune rejection or promoter shutoff. Furthermore, when an AAV2 vector carrying human δ-sarcoglycan gene was similarly delivered into the heart of Bio14.6 Syrian hamster, a congestive heart failure and limb girdle muscular dystrophy animal model, widespread therapeutic gene transfer was achieved in a majority of the cardiomyocytes. Efficient expression of the human δ-sarcoglycan gene in the dystrophic hamster hearts restored the entire sarcoglycan complex that was missing due to the primary deficiency of δ-sarcoglycan. Transgene expression persisted for 4 months (the duration of the study) without immune rejection or promoter shutoff. These results indicate that AAV is a promising vector system for cardiac gene therapy.
Gene Therapy (2003) **10**, 1807–1813. doi:10.1038/sj.gt.3302078

Keywords: *AAV vector; δ-sarcoglycan; heart; Bio14.6 hamster*

Introduction

Cardiovascular diseases are the leading cause of death in the developed countries. Other related ailments such as hereditary cardiomyopathies are also a common cause of morbidity and mortality. Gene therapy has been extensively studied as a novel strategy for heart diseases. Owing to the quiescent nature of cardiomycytes, the classic retrovirus vectors are not suitable for intracardiac gene transfer. On the other hand, adenovirus (Ad) vectors and naked plasmid DNA have been successfully used to deliver genes into the heart tissue. Ad vectors could be delivered into the myocardium either by direct intramyocardial injection[1] or through coronary circulation.[2] However, a major limitation is the vector-related immune responses in immunocompetent recipients. In addition, it is extremely difficult to deliver Ad vectors through coronary circulation, because of the large viral particle size that hinders the virus from exiting the blood vessel and infecting cardiomyocytes. Direct injection of naked DNA into myocardium could achieve gene transfer but with very limited efficiency.[3] Adeno-associated virus (AAV) vectors are based on nonpathogenic and replication-defective parvoviruses, which have small viral particle sizes (~22 nm in diameter).[4] AAV vectors have been widely used for efficient gene delivery into a variety of tissues *in vivo* including brain,[5] liver,[6,7] lung,[8] muscle,[9,10] and eye.[11,12] Currently, AAV vectors are the most promising gene delivery system in muscle tissues due to its efficient, widespread and persistent gene transfer. Although extensive studies have been carried out using AAV vectors in skeletal muscles for both muscular dystrophies[13–18] and for metabolic diseases,[19–22] few studies involved cardiac muscles.

Limb girdle muscular dystrophies are a group of heterogeneous inherited neuromuscular diseases.[23] The severe and early-onset phenotypes are usually caused by mutations in sarcoglycan (SG) genes α (LGMD 2D), β (LGMD 2E), γ (LGMD2C), and δ (LGMD 2F). These small transmembrane proteins associate in equal stoichiometry on muscle cell membrane to form a heterotetramer, named SG complex.[24] Recently, sarcospan was also identified as a member of the SG complex.[25] Primary deficiency of any single SG protein can result in partial or complete disappearance of the SG complex on the sarcolemma, leading to muscular dystrophy and cardiomyopathy. The cardiomyopathy Syrian hamster Bio14.6[26] was the first available LGMD 2F animal model, with a large deletion in its δ-sarcoglycan gene.[27,28] The genetic mutation causes biochemical deficiency of the entire SG complex on both skeletal and cardiac muscle

Correspondence: Dr X Xiao, Department of Molecular Genetics and Biochemistry, Room W1244 BST, University of Pittsburgh, Pittsburgh, PA 15261, USA
Received 22 January 2003; accepted 07 April 2003

cell membranes. In addition to skeletal muscle myopathy, the Bio14.6 hamsters also suffer from severe cardiomyopathy and congestive heart failure, a primary cause of premature death. As a result of the resemblance to human patients, the Bio14.6 hamster provides an excellent animal model of gene therapy for both skeletal and cardiac deficiencies. Successful gene therapy studies on skeletal muscle in Bio14.6 hamsters have been achieved using both Ad vector[29] and AAV vectors carrying δ-sarcoglycan gene,[14,15,18] which restored the missing sarcoglycan complex on the muscle cell membrane, improved both muscle histopathology and myofiber membrane integrity and, more importantly, recovered the muscle contractile force deficits.[15] In addition, direct local injection of an AAV vector carrying δ-sarcoglycan gene into the myocardium of Bio14.6 hamster has been recently reported.[30] However, AAV vector-mediated SG gene delivery through the coronary artery into the entire myocardium has not been reported.

In this study, we have used AAV2 vectors carrying either a reporter Lac-Z gene or the δ-sarcoglycan gene into the myocardium of the hamsters in a heterotopic heart transplant model. Highly efficient and persistent transgene expression and biochemical restoration of the δ-sarcoglycan and the SG complex in the hamster hearts have been accomplished.

Results

Widespread and persistent transgene expression in cardiomyocytes after coronary delivery of AAV-LacZ vector

Cardiomyopathy in the Bio14.6 hamster is caused by the lack of δ-sarcoglycan in the cardiomyocytes. To correct the genetic deficiency, widespread and persistent expression of the therapeutic gene into a majority of the cardiomyocytes is desirable. Direct intramyocardium injection of the vectors apparently could not render widespread gene transfer throughout the heart. To investigate whether highly efficient gene transfer can be achieved by the coronary circulation, we initially tested an AAV-Lac-Z reporter vector in a heterotopic heart transplantation model.[31] The reason to choose the *ex vivo* heart transplant method instead of the *in situ* heart gene transfer is mainly because of the technical difficulty of the latter method in small rodents.

To test and compare the transduction efficiency of AAV2 and Ad5 vectors in hamster hearts, healthy F1B hamster donor hearts were infused *ex vivo* through coronary circulation with 200 μl of AAV-CMV-Lac-Z or Ad-CMV-LacZ virus (1×10^{12} viral genomes, v.g.), and then transplanted into the abdomen of recipients of the same strain. A 2 weeks after vector delivery, X-gal staining of the AAV vector-treated heart showed robust and widespread LacZ gene expression in a vast majority of the cardiomyocytes (Figure 1a), while the Ad vector-treated heart showed inefficient gene transfer with few LacZ-positive cardiomyocytes (Figure 1b). Cross-sectioning of the whole heart 1 year after AAV gene transfer revealed LacZ-positive myocytes in up to 90% of the heart tissue (Figure 1c). A preference of gene transfer by the AAV2 vectors in peripheral areas over the central areas of the myocardium was occasionally observed (data not shown; Figure 3). This might be due to the differences in blood vessel densities in those muscles. No signs of immune cell infiltration and immune rejection of AAV-LacZ-transduced myocytes were observed. These results demonstrated that the AAV vector could be effectively delivered into the myocardium through

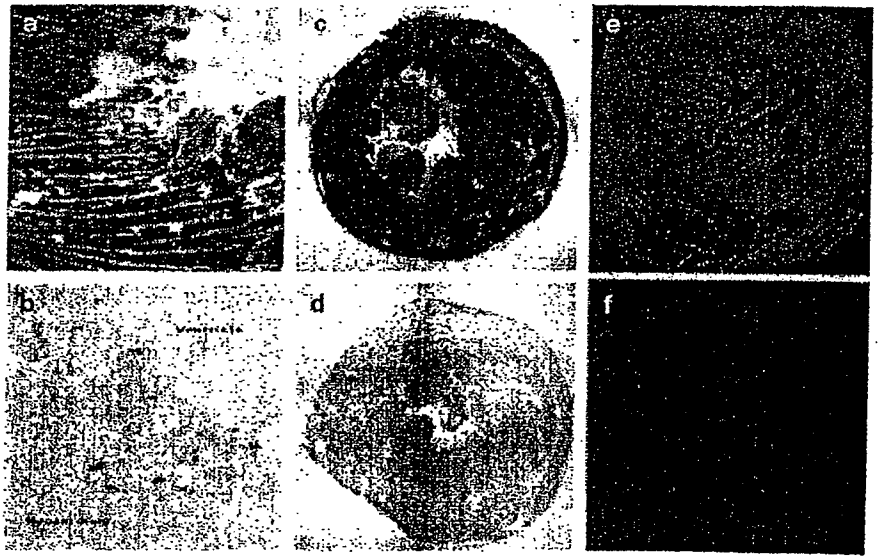


Figure 1 AAV vector-mediated efficient intracardiac (intramyocardium) gene transfer through coronary circulation in hamster hearts. Normal hamster hearts of F1B strain were infused either with AAV-CMV-LacZ vector (a) or Ad-CMV-LacZ vector (b), and stained by X-gal and H&E on the cryo-thin-sections of the transplanted hearts 2 weeks after vector infusion (photographs were taken with a ×20 lens). AAV-CMV-LacZ also conferred long-term, intracardiac gene expression for 1 year (c), while the control heart without gene transfer showed no X-gal staining (d) (photographs of the whole-heart cross-section were taken with a ×4 lens and spliced together). The dystrophic Bio14.6 hamster hearts were infused with AAV-CMV-δSG vector and immunofluorescently stained with anti-δ-SG antibody and Cy3-labeled secondary antibody (e) at 4 months after vector infusion (photograph of the whole-heart cross-section was taken with a ×2 lens). The untreated Bio14.6 heart showed no fluorescent staining (f) (photograph was taken with a ×20 lens).

coronary artery circulation. The AAV particles were small enough to exit the capillary blood vessel without additional hydrostatic pressure.

Efficient AAV-δ-sarcoglycan gene transfer in cardiomyocytes and restoration of sarcoglycan complex

Having succeeded in normal hamster heart with a reporter gene, we next investigated whether the therapeutic gene δ-sarcoglycan could also be efficiently transduced into the hearts of the diseased Bio14.6 hamsters by AAV2. In a similar manner, hearts isolated from Bio14.6 hamster donors were infused *ex vivo* via coronary circulation with 200 µl of AAV-δ-sarcoglycan (1×10^{12} viral genomes, v.g.) and then transplanted into the abdomen of Bio14.6 hamster recipients. As the average lifespan of the Bio14.6 hamsters was reportedly 5 months,[26] the AAV vector-treated and heterotopically transplanted hearts were examined for transgene expression at 4 months after gene transfer (5 months of age). Immunofluorescent staining was performed on the cross-sections of the vector-transduced hearts with a monoclonal antibody specific to human δ-sarcoglycan, which was the AAV transgene product in this study. The results showed extensive gene expression in a majority of the cardiomyocytes in the entire heart (Figure 1e). Similar to our previous studies in the skeletal muscles,[14,15] AAV-δ-sarcoglycan gene transfer resulted in overexpression of the protein in some of the myocytes, and revealed cytoplasmic staining as well as membrane staining of the protein (Figure 2d and d′). However, overexpression of the δ-sarcoglycan did not interfere with the restoration of the other three SG components (α-, β-, and γ-sarcoglycans) onto the plasma membrane of the cardiomyocytes (Figure 2a–c). No cytoplasmic staining of α-, β-, and γ-sarcoglycans in AAV vector-transduced myocytes was observed, which is again consistent with the results obtained in skeletal muscle gene transfer.[14,15] Thus, expression of δ-sarcoglycan in the Bio14.6 hamster hearts effectively restored the missing SG complex.

Protection of cardiomyofiber plasma membrane integrity

The restoration of the SG complex should also render a protective effect on the cardiomyocyte cell membrane integrity and prevent membrane leakage. To examine the protective effect, Evans blue dye (a low molecular weight dye with red fluorescence) was injected into the Bio14.6 hamsters treated with AAV-δ-sarcoglycan. Examination of the cardiac muscle showed exclusion of Evans blue dye in the sarcoglycan-positive heart tissue (Figure 3, left half, β-sarcoglycan staining on the cell membrane) and leakage into the myocytes in the areas without AAV gene transfer (Figure 3, right half, red fluorescence of Evans blue dye in the cytoplasm of leaking cardiomyocytes), indicating a protective effect on plasma membrane integrity by the SG gene therapy.

Inefficient transgene expression in blood vessels

Since the AAV vectors were delivered into the myocardium through coronary circulation and robust transgene expression was observed in cardiomyocytes, we wished to examine whether the blood vessels were also transduced. Cross-sections of hearts transduced by the AAV-LacZ vector were analyzed for transgene expression in the blood vessels, which were surrounded by highly transduced cardiomyocytes to assure that those blood vessels also had been exposed to the AAV vectors.

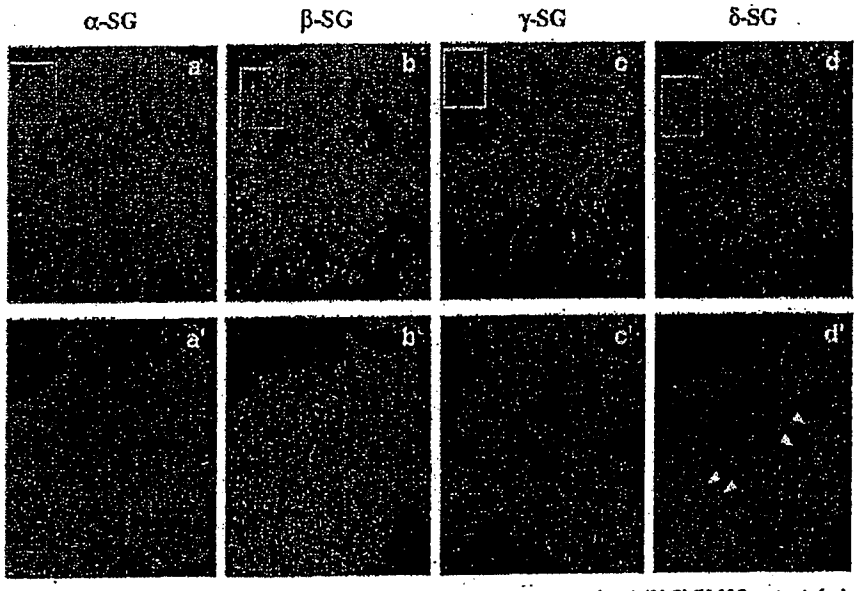


Figure 2 Restoration of the missing SG protein complex on the cell membrane of cardiomyocytes after AAV-CMV-δSG vector infusion in Bio14.6 hamster hearts. Immunofluorescent staining of the cryo-thin-sections with anti-δ-SG antibody showed widesprend δ-SG gene expression (d and d′) and restoration of the other three sarcoglycans, α-SG (a and a′), β-SG (b and b′), and γ-SG (c and c′). Note the over-expression and accumulation of δ-SG in the cytoplasm of a number of myocytes (d and d′, arrows). Photographs of panels a–d were taken with a ×4 lens, while panels a′–d′ were the enlargement of the highlighted areas of panels a–d to show membrane staining of the SG proteins.

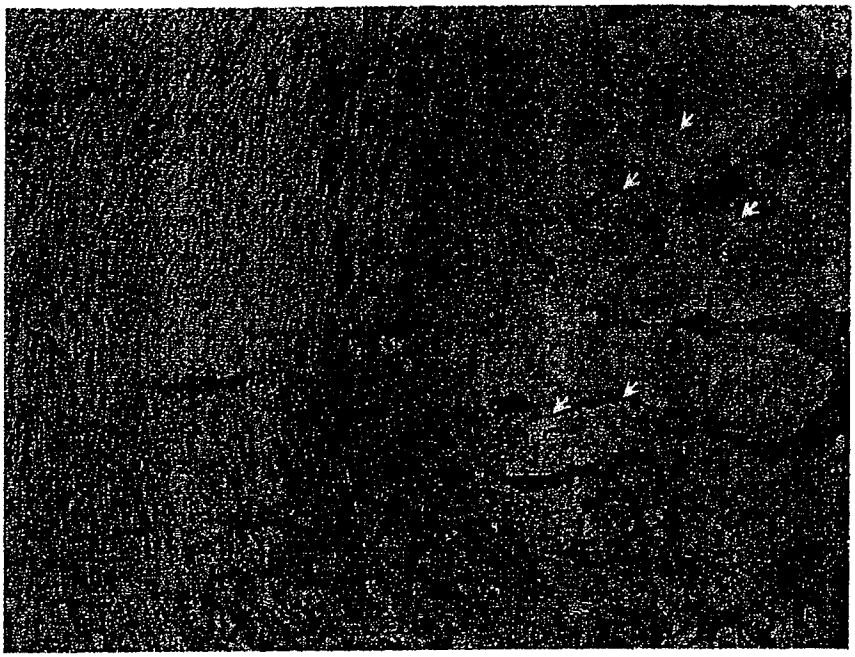


Figure 3 Protection of myocyte membrane integrity by AAV-CMV-δSG gene transfer. The hamster was injected intravenously with Evans blue dye to examine the myocyte plasma membrane integrity. Cryo-thin-section of vector-treated heart was immunofluorescently stained with anti-β-SG antibody, showing the restoration of the SG complex on the cell membrane (left portion of the photograph, taken with a × 4 lens) and prevention of dye leakage into those cells. By contrast, the areas without gene transfer and negative for β-SG staining (right portion of the photograph) had numerous myocytes leaked and filled with Evans blue dye (it has strong red fluorescence) in the entire cytoplasm, as highlighted by arrows. Note: As δ-SG showed some cytoplasm accumulation (see Figure 2), the anti-β-SG antibody was instead used to assure that the red cytoplasm fluorescence was a result of Evans blue dye leakage.

Surprisingly, no X-gal staining could be observed in blood vessels of various sizes in the AAV-LacZ-treated hamster hearts, although their neighboring myocytes were all highly transduced and showed strong X-gal staining (Figure 4a and b). These results suggested that AAV-2 vectors were inefficient in delivering transgene expression in cardiac blood vessels.

Discussion

Previously, efficient gene transfer and therapeutic benefits have been demonstrated in the skeletal muscles of the dystrophic Bio14.6 hamsters after gene therapy with the AAV-δ-sarcoglycan vectors.[14,15,18] In this study, we showed that coronary infusion of AAV vectors into the hearts is an efficient method of gene transfer into the myocardium. Widespread and sustained transgene expression in the hearts of both healthy and dystrophic hamsters was accomplished. AAV-δ-sarcoglycan gene transfer also restored the entire missing SG complex, whose deficiency is the primary cause of the cardiomyopathy and congestive heart failure in the Bio14.6 hamsters as well as in some sarcoglycanopathy patients.

Intracardiac gene transfer has been one of the major emphases in the field of gene therapy. Both Ad vectors and naked plasmid DNA vectors have been extensively studied for intracardiac gene transfer. These vectors have also been used in clinical trials of cardiovascular diseases to deliver therapeutic genes that promote local angiogenesis in the ischemic heart tissues.[1,3] For that purpose, limited local gene transfer may be sufficient to render therapeutic effects. However, for hereditary cardiomyopathies and other heart conditions where extensive gene transfer is required, adenoviral and nonviral vectors would face formidable hurdles to achieve widespread transgene expression. When delivered through blood circulation, both Ad vectors and naked plasmid DNA were not only hindered by their size constraint in exiting the capillary blood vessels, but also limited by the extracellular matrix barrier[32,33] and the lack of receptors for naked DNA on the cardiomyocytes. Recently, Ad vectors carrying Lac-Z reporter gene and δ-sarcoglycan gene were delivered into the heart of normal and Bio14.6 hamster hearts after transient aortic occlusion.[2] To achieve efficient intracardiac gene transfer, both hypothermia and cardioplegia were also required. In addition, the use of histamine in the coronary circulation for several minutes before Ad delivery was essential to achieve sufficient blood vessel fenestration and leakage to allow the Ad to enter the extravascular space.[2]

By contrast, AAV vectors have much smaller particle sizes (~22 nm in diameter) than the Ad (~100 nm in diameter including fibers). As a result, AAV vectors can bypass the blood vessel pores and extracellular matrix with less difficulty. In fact the vectors have been investigated for intracardiac gene transfer by means of both coronary circulation and direct intramyocardium injection. An early study in pig hearts with an AAV-LacZ vector delivered by a catheter into a branch of the coronary artery achieved transgene expression in large numbers but only a small overall percentage of cardio-

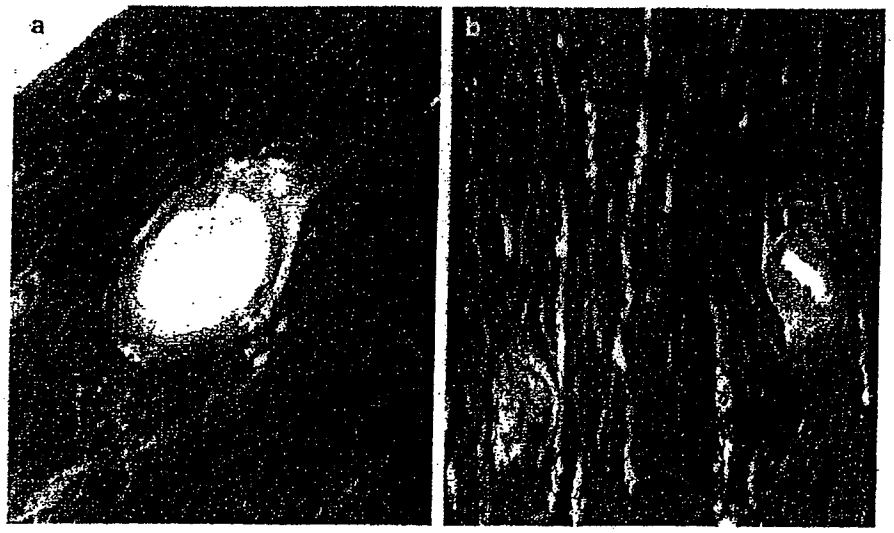


Figure 4 Inefficient gene transfer and transgene expression in blood vessels. Thin sections of AAV-CMV-LacZ vector-treated hearts were stained with X-gal and H&E. Although the surrounding cardiomyocytes were efficiently transduced by the AAV vector and showed strong X-gal staining of blue color, the blood vessels of either large size (a) or small size (b) however showed no detectable LacZ gene expression.

mycytes,[34] probably due to insufficient vector doses. Direct intramyocardium injection of AAV vectors also rendered efficient local gene transfer in hamster heart and therapeutic benefits in the TO-2 hamster,[30] a derivative of the cardiomyopathy Bio14.6 hamsters. In our study, AAV vectors were directly infused through the aorta into the coronary artery without extra pressure and without the use of histamine or blood vessel dilating chemicals. Highly efficient and widespread gene transfer in the whole heart indicated that AAV viral particles were small enough to exit the capillary blood vessels and infect the cardiomyocytes. Under the same *ex vivo* condition, an adenovirus LacZ vector, however, failed to achieve significant gene transfer (Figure 1b). The use of histamine and papaperin was necessary for efficient gene transfer in the same *ex vivo* heart transplant model when an Ad vector was used.[18] Given the smaller particle sizes, AAV vectors should face less difficulties than the Ad vectors.[34–36]

Owing to the technical difficulty for *in situ* gene delivery into the hearts of small rodents via coronary circulation, we employed the heterotopic heart transplant, a widely used model in the transplant field, to examine AAV-mediated gene transfer in the cardiomyopathic Bio14.6 hearts. Although widespread δ-sarcoglycan gene transfer and restoration of the entire SG complex were accomplished in the vector-treated hearts, it is difficult to evaluate the physiological functions of such a heart because the transplanted heart did not have normal blood circulation in the left ventricle. This makes echocardiograph and hemodynamics evaluations non-applicable. An improved transplant model should offer a solution to this problem.[37] In addition, the lack of mechanical stress to the heart may also minimize the contraction-induced damage to the dystrophic heart muscle, resulting in less severe phenotypes. Nonetheless, we still observed the Evans blue dye leakage into the myocytes in the areas that lacked δ-sarcoglycan gene transfer, and the protection of myocytes membrane integrity in the area that showed δ-sarcoglycan gene transfer (Figure 3). As a result the high percentage of therapeutic gene transfer in the cardiomyocytes, a recovery in physiological functions would almost be certain if the heart were in its *in situ* position. This notion is supported by two previous studies, where either a direct injection of left ventricle muscle with an AAV-δ-sarcoglycan vector[30] or a coronary infusion with an Ad-δ-sarcoglycan vector[2] have achieved significant physiological function recovery. The AAV vector treatment also slightly prolonged the lifespan of the cardiomyopathic TO-2 hamsters even though only a portion of the left ventricle received gene transfer by a single local injection of the δ-sarcoglycan vector. To achieve clinical relevance, *in situ* perfusion of the diseased heart has to be performed, which is technically difficult but feasible.

Finally, it is also interesting to note the lack of detectable transgene expression in the blood vessels of the AAV vector-treated heart, both with the LacZ reporter gene or with the δ-sarcoglycan gene (data not shown), despite the extensive transgene expression in the surrounding myocytes. This phenomenon may be a result of inefficient infection of blood vessel cells by AAV2-based vectors, which could be possibly limited by the lack of receptor[38] and coreceptors,[39,40] impaired intracellular trafficking, and inability of viral DNA second-strand DNA synthesis. Alternatively, it may also be the result of promoter shutoff in those cells. Given the implication of blood vessel deficiency in some sarcoglycanopathy-related heart failure,[41] it would be interesting to see whether extensive gene correction in the cardiomyocytes, rather than the cardiovasculature, would render a complete or a partial therapeutic efficacy. A combination of gene therapy and drug therapy[42] may be required to achieve maximal benefits.

Materials and methods

Production of AAV vectors

Construction of AAV vectors containing human δ-sarcoglycan cDNA or β-galactosidase (Lac-Z) gene under the control of CMV promoter (AAV-CMV-δSG) has been previously described (Li). The recombinant viral vector stocks were produced by cotransfection methods as described by Xiao *et al*.[43] The AAV viral vectors were purified twice through CsCl density gradient purification according to the previously published protocols.[9,44] The vector titers of viral genome particle number were determined by DNA dot blot method,[44] and were in the range of 5×10^{12} viral genome (v.g.) particles per ml. An adenovirus CMV-LacZ vector was made by the standard protocol[9] and the vector titer was 5×10^{12} viral genome particles per ml.

Coronary artery delivery of AAV and Ad vectors

Healthy F1B and dystrophic Bio14.6 hamsters were purchased from Bio Breeders (Fitchburg, MA, USA) and handled in accordance with the institutional guidelines of the University of Pittsburgh. Heterotopic heart transplantation was done on 1–2-month-old animals. The method for heterotopic heart transplantation was adapted from the rat procedure of Ono and Lindsey.[31] All animals were anesthetized with tribromoethanol (Avertin®, 250 mg/kg i.p.). The heart was transplanted into the abdomen with end-to-side anastomosis of aorta to aorta and pulmonary artery to vena cava. For *ex vivo* intravascular vector injection into the donor heart after harvest, cold AAV2-CMV-LacZ vector or AAV2-CMV-dSG (0.2 ml in DMEM media) or Ad5-CMV-LacZ (0.2 ml in PBS saline) was quickly infused into the aorta, which was clamped so that the vector solution would flow through the coronary circulation and irrigate the entire myocardium. No recirculation of the vector solution was performed. The heart was soaked in cold saline for 5–10 min and then transplanted into the abdominal cavity of the syngeneic recipients. The heart graft contraction was monitored weekly by palpation. AAV-CMV-LacZ gene expression was detected by X-gal staining, while AAV-CMV-dSG gene expression was detected by immunofluorescent staining of the cryostat thin sections of the vector-treated hearts. To test the cardiomyocyte cell membrane integrity *in vivo*, Evans blue dye (10 mg/ml PBS) was injected intravenously into Bio14.6 hamsters at 0.1 mg/g of body weight. The animals were killed 12 h after dye injection. Vector-treated hearts were collected and cryosectioned. Evans blue dye positive cardiomyocytes, which showed red fluorescence in the cytoplasm, were observed under the fluorescent microscope with Rhodamine filters.

Immunofluorescent staining

Cryostat sectioning of the heart tissue was performed at 5 μM thickness with a Leica microtome. For immunofluorescent staining,[15] the unfixed muscle cryosections were immediately blocked in 10% horse serum and PBS at room temperature for 1 h. Monoclonal antibodies against α-, β-, γ-, and δ-sarcoglycan SGs (Novocatra Laboratories) were diluted 1:100 in 10% horse serum/PBS, and incubated with the cryosections for 2 h at room temperature. After three washes, the sections were incubated with Cy-3-labeled anti-mouse antibody at 1:500 dilution in 10% horse serum/PBS (Jackson Immuno Research Laboratories). After three washes, the samples were mounted in Gelmount (Fisher). Photographs were taken with a Nikon fluorescent microscope equipped with a digital camera.

Acknowledgements

We thank Michael Xiao for critical reading of the manuscript. This work is supported by NIH Grants AR 45967 and AR45925 and a grant from Chinese National Science Foundation.

References

1 Harvey BG *et al*. Safety of local delivery of low- and intermediate-dose adenovirus gene transfer vectors to individuals with a spectrum of morbid conditions. *Hum Gene Ther* 2002; 13: 15–63.
2 Ikeda Y *et al*. Restoration of deficient membrane proteins in the cardiomyopathic hamster by *in vivo* cardiac gene transfer. *Circulation* 2002; 105: 502–508.
3 Losordo DW *et al*. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation* 1998; 98: 2800–2804.
4 Xie Q *et al*. The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc Natl Acad Sci USA* 2002; 99: 10405–10410.
5 Kaplitt MG *et al*. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat Genet* 1994; 8: 148–154.
6 Ponnazhagan S *et al*. Adeno-associated virus 2-mediated gene transfer *in vivo*: organ-tropism and expression of transduced sequences in mice. *Gene* 1997; 190: 203–210.
7 Xiao W *et al*. Adeno-associated virus as a vector for liver directed gene therapy. *J Virol* 1998; 72: 10222–10226.
8 Flotte TR *et al*. Stable *in vivo* expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc Nat Acad Sci USA* 1993; 90: 10613–10617.
9 Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 1996; 70: 8098–8108.
10 Kessler PD *et al*. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc Nat Acad Sci USA* 1996; 93: 14082–14087.
11 Ali RR *et al*. Gene transfer into the mouse retina mediated by an adeno-associated viral vector. *Hum Mol Genet* 1996; 5: 591–594.
12 Flannery JG *et al*. Efficient photoreceptor-targeted gene expression *in vivo* by recombinant adeno-associated virus. *Proc Nat Acad Sci USA* 1997; 94: 6916–6921.
13 Cordier L *et al*. Rescue of skeletal muscles of gamma-sarcoglycan-deficient mice with adeno-associated virus-mediated gene transfer. *Mol Ther* 2000; 1: 119–129.
14 Li J *et al*. rAAV vector-mediated sarcogylcan gene transfer in a hamster model for limb girdle muscular dystrophy. *Gene Therapy* 1999; 6: 74–82.
15 Xiao X *et al*. Full functional rescue of a complete muscle (TA) in dystrophic hamsters by adeno-associated virus vector-directed gene therapy. *J Virol* 2000; 74: 1436–1442.
16 Wang B, Li J, Xiao X. Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. *Proc Natl Acad Sci USA* 2000; 97: 13714–13719.
17 Watchko J *et al*. Adeno-associated virus vector-mediated minidystrophin gene therapy improves dystrophic muscle

contractile function in mdx mice. *Hum Gene Ther* 2002; 13: 1451–1460.
18 Greelish JP *et al*. Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector. *Nat Med* 1999; 5: 439–443.
19 Chao H *et al*. Persistent expression of canine factor IX in hemophilia B canines. *Gene Therapy* 1999; 6: 1695–1704.
20 Kay MA *et al*. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; 24: 257–261.
21 Song S *et al*. Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. *Proc Natl Acad Sci USA* 1998; 95: 14384–14388.
22 Fraites Jr TJ *et al*. Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. *Mol Ther* 2002; 5: 571–578.
23 Hoffman EP. Counting muscular dystrophies in the post-molecular census. *J Neurol Sci* 1999; 164: 3–6.
24 Lim LE, Campbell KP. The sarcoglycan complex in limb-girdle muscular dystrophy. *Curr Opin Neurol* 1998; 11: 443–452.
25 Crosbie RH *et al*. Sarcospan, the 25-kDa transmembrane component of the dystrophin–glycoprotein complex. *J Biol Chem* 1997; 272: 31221–31224.
26 Homburger F, Baker JR, Nixon CW, Whitney R. Primary, generalized polymyopathy and cardiac necrosis in an inbred line of Syrian hamsters. *Med Exp* 1962; 6: 339–345.
27 Nigro V *et al*. Identification of the Syrian hamster cardiomyopathy gene. *Hum Mol Genet* 1997; 6: 601–607.
28 Sakamoto A *et al*. Both hypertrophic and dilated cardiomyopathies are caused by mutation of the same gene, delta-sarcoglycan, in hamster: an animal model of disrupted dystrophin-associated glycoprotein complex. *Proc Nat Acad Sci USA* 1997; 94: 13873–13878.
29 Holt KH *et al*. Functional rescue of the sarcoglycan complex in the BIO 14.6 hamster using delta-sarcoglycan gene transfer. *Mol Cell* 1998; 1: 841–848.
30 Kawada T *et al*. Rescue of hereditary form of dilated cardiomyopathy by rAAV-mediated somatic gene therapy: amelioration of morphological findings, sarcolemmal permeability, cardiac performances, and the prognosis of TO-2 hamsters. *Proc Natl Acad Sci USA* 2002; 99: 901–906.
31 Ono K, Lindsey ES. Improved technique of heart transplantation in rats. *J Thorac Cardiovasc Surg* 1969; 57: 225–229.
32 Pruchnic R *et al*. The use of adeno-associated virus to circumvent the maturation-dependent viral transduction of muscle fibers. *Hum Gene Ther* 2000; 11: 521–536.
33 Huard J *et al*. The basal lamina is a physical barrier to herpes simplex virus-mediated gene delivery to mature muscle fibers. *J Virol* 1996; 70: 8117–8123.
34 Kaplitt MG *et al*. Long-term gene transfer in porcine myocardium after coronary infusion of an adeno-associated virus vector. *Ann Thorac Surg* 1996; 62: 1669–1676.
35 Hoshijima M *et al*. Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via *in vivo* cardiac rAAV gene delivery. *Nat Med* 2002; 8: 864–871.
36 Asfour B *et al*. Uniform long-term gene expression using adeno-associated virus (AAV) by *ex vivo* recirculation in rat-cardiac isografts. *Thorac Cardiovasc Surg* 2002; 50: 347–350.
37 Asfour B *et al*. A simple new model of physiologically working heterotopic rat heart transplantation provides hemodynamic performance equivalent to that of an orthotopic heart. *J Heart Lung Transplant* 1999; 18: 927–936.
38 Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 1998; 72: 1438–1445.
39 Summerford C, Bartlett JS, Samulski RJ. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med* 1999; 5: 78–82.
40 Qing K *et al*. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat Med* 1999; 5: 71–77.
41 Coral-Vazquez R *et al*. Disruption of the sarcoglycan–sarcospan complex in vascular smooth muscle: a novel mechanism for cardiomyopathy and muscular dystrophy. *Cell* 1999; 98: 465–474.
42 Cohn RD *et al*. Prevention of cardiomyopathy in mouse models lacking the smooth muscle sarcoglycan–sarcospan complex. *J Clin Invest* 2001; 107: R1–R7.
43 Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 1998; 72: 2224–2232.
44 Snyder R, Xiao X, Samulski RJ. Production of recombinant adeno-associated viral vectors. In: Dracopoli N *et al* (eds). *Current Protocols in Human Genetics*. John Wiley & Sons Ltd.: New York, 1996, pp 12.11.11–12.12.23.

Artikel

Zeitschrift für Kardiologie
Verlag: Steinkopff Verlag
ISSN: 0300-5860 (Paper) 1435-1285 (Online)
DOI: 10.1007/s00392-004-0008-1
Heft: Band 93, Nummer 3
Datum: März 2004
Seiten: 171 - 193

REVIEW

Nucleic acid-based modulation of cardiac gene expression for the treatment of cardiac diseases Approaches and perspectives

W. Poller[1] ✉, H. Fechner[1], J. Kurreck[2], M. Pauschinger[1], U. Kühl[1], V. A. Erdmann[2], J. M. J. Lamers[3] and H.-P. Schultheiss[1]

(1) Department of Cardiology and Pneumology, University Hospital Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany

(2) Institute for Biochemistry, Freie Universität, Berlin, Germany

(3) Department of Biochemistry, Cardiovascular Research Institute COEUR, Erasmus University, Rotterdam, The Netherlands

Received: 7 April 2003 **Accepted:** 4 August 2003

Summary. During the past few years major conceptual and technical advances have been made towards the therapeutic modulation of cardiac gene expression for the treatment of cardiac diseases. Among these are 1) the identification of new molecular therapy targets in cardiac disorders, often derived from genetic animal models. 2) A better understanding of the molecular and cellular determinants of cardiac gene transfer *in vivo*, in animal models and in first clinical trials. 3) The development of novel regulatable and long-term stable vector systems. This review is focused on nucleic acid-based modulation of cardiac calcium homeostasis as a paradigm for the new gene therapeutic approaches, since recent landmark papers have suggested this to be a molecular target of key importance in heart failure. In particular, the development of severe heart failure in the genetic $MLP^{-/-}$ animal model could be completely abolished by the targeted ablation of phospholamban (PL), a key regulator of cardiac calcium homeostasis. This impressive effect of permanent germline PL ablation provides—in conjunction with former important work on disturbed calcium handling in the failing human heart—a rationale for the gene therapeutic approach of *ad hoc* suppression of PL by *antisense* strategies (*antisense* RNAs, ribozymes, RNA *interference*) or PL variants. Based on the broad spectrum of methods employed to characterize this general strategy, PL-targeted approaches may be considered as a paradigm of future genetic treatments of cardiac disorders,

EXHIBIT X

although the differences between animal models and humans must be kept in mind. High safety of any such therapy will be a prerequisite for any possible clinical application and therefore novel methods to improve control are being devised: 1) The regulation of gene therapy vectors by biochemical abnormalities associated with the target disease itself ("*Induction-by-Disease*" gene therapy). 2) External control of vector activity by the employment of drug-sensitive promotors. In addition, the important goal of cardiac long-term stability of the therapeutic vectors has recently been achieved in animal models using vectors derived from adeno-associated viruses (AAVs).

Key words Antisense RNA - cardiac calcium homeostasis - gene therapy - heart failure - phospholamban - RNA interference - ribozymes

---

Nucleinsäure-basierte Modulation der kardialen Genexpression zur Behandlung kardialer Erkrankungen

Zusammenfassung. In den letzten Jahren wurden wichtige konzeptionelle und technische Fortschritte erzielt auf dem Weg zur therapeutischen Modulation der kardialen Genexpression bei kardialen Erkrankungen. Zu diesen Fortschritten zählt 1. Die Identifikation neuer therapeutischer Ziele bei der Herzinsuffizienz, oft anhand genetischer Tiermodelle. 2. Ein besseres Verständnis der molekularen und zellulären Determinanten kardialen Gentransfers *in vivo*, in Tiermodellen und in ersten klinischen Studien. 3. Die Entwicklung regulierbarer und langzeitstabiler Vektorsysteme. Diese Übersicht fokussiert sich auf die Nukleinsäure-basierte Modulation der kardialen Calcium- Homöostase als ein Paradigma für diese gentherapeutischen Ansätze, da jüngere bahnbrechende Arbeiten annehmen lassen, dass die Calcium-Homöostase ein Therapieziel von zentraler Bedeutung bei der Herzinsuffizienz darstellt. So konnte die Entwicklung einer schweren Herzinsuffizienz im genetischen MLP$^{-/-}$ Tiermodell vollständig blockiert werden durch die gezielte genetische Ausschaltung des Phospholambans (PL), eines zentralen Regulators der kardialen Calcium-Homöostase. Dieser eindrucksvolle Effekt einer permanenten PL-Ausschaltung liefert—in Verbindung mit wichtigen früheren Arbeiten über Störungen der Calcium-Homöostase im insuffizienten menschlichen Herzen—eine rationale Basis für den therapeutischen Ansatz einer *ad hoc*-Suppression von PL mit Hilfe von *antisense*-Strategien (*antisense*-RNAs, Ribozyme, RNA-*Interferenz*) oder PL-Varianten. Wegen des sehr breiten Spektrums an Methoden, die zur Charakterisierung dieser Strategie eingesetzt worden sind, können PL-gerichtete Strategien als Paradigma für zukünftige genetische Therapien kardialer Erkrankungen angesehen werden, trotz bekannter komplexer Unterschiede zwischen Tiermodellen und dem Menschen. Hohe Sicherheit jeglicher solchen Therapie ist eine Voraussetzung für ihre mögliche klinische Anwendung und neue Kontrollmethoden werden daher entwickelt: 1. Regulation des Vektors durch biochemische Anomalien, die mit der Zielkrankheit selbst assoziiert sind („*Inductionby- Disease*" Gentherapie). 2. Externe Kontrolle der Vektoraktivität durch den Einsatz pharmakon- sensitiver Promotoren. Darüber hinaus konnte das wichtige Ziel kardialer Langzeit-Stabilität der therapeutischen Vektoren in jüngster Zeit tierexperimentell mit Vektoren auf der Basis von Adeno-assoziierten Viren erreicht werden.

Schlüsselwörter Antisense-RNA - kardiale Calcium-Homöostase - Gentherapie - Herzinsuffizienz - Phospholamban - RNA-Interferenz - Ribozyme

W. Poller
Email: poller@ukbf.fu-berlin.de
Phone: +49-(0)30-84 45-42 34
Fax: +49-(0)30-84 45-46 48

Die Referenzen dieses Artikels sind fuer Abonnenten gesichert.